**Université de Fribourg (Suisse)**

**Département de Biologie**

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# Regulation of TOR Complex I and Cell Growth by Amino Acids in *Saccharomyces cerevisiae*

THESE

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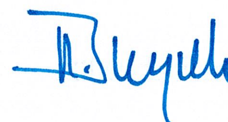

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To whom it may concern,

I, Grégory Bonfils, herein declare that I wrote this thesis work on my own, based on a personal scientific work without illicit help.

“*Οἶδα οὐδὲν εἰδώς*” (Socrate)
“*Je sais que je ne sais rien*”

*"La chance ne sourit qu’aux esprits bien préparés "* (Louis Pasteur)

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List of abbreviations:

AMP: Adenosine MonoPhosphate
ADP: Adenosine DiPhosphate
ATP: Adenosine TriPhosphate
ATPase: Adenosine TriPhosphatase
cAMP: cyclic AMP
AVT: Amino acid Vacuolar Transport
BCAA: Branched-Chain Amino Acid
CHX: CycloHeXimide
CLS: Chronological LifeSpan
CP1: Connective Peptide 1
CTD: C-Terminal Domain
EGOC: Exit from a rapamycin-induced GrOwth arrest Complex
ER: Endoplasmic Reticulum
GAAC: General Amino Acid Control
GABA: Gamma Amino Butyric Acid
GAP: GTPase Activating Protein
GEF: Guanine nucleotide Exchange Factor
GDP: Guanosine DiPhosphate
GTP: Guanosine TriPhosphate
GTPase: Guanosine TriPhosphatase
HEK: Human Embryonic Kidney
HM: Hydrophobic Motif
HPLC: High performance Liquid Chromatography
MPA: MycoPhenolic Acid
MSX: Methionine Sulfoximine
NCR: Nitrogen Catabolite Repression
ORF: Open Reading Frame
uORF: upstream ORF
Pi: inorganic phosphate
PIKK: Phosphatidyl Inositol-Kinase Kinase
PP2A: Protein Phosphatase 2A
RNA: RiboNucleic Acid
tRNA: transfer RNA
mRNA: messenger RNA
Ribi: Ribosome biogenesis
RP: Ribosomal Protein
RTG: ReTroGrade
SCF: Skp, Cullin, F-box
TAP: Tandem Affinity Purification
TCA: TriCarboxylic Acid
TM: Turn Motif
TORC1: Target Of Rapamycin Complex 1
mTORC1: mammalian TORC1
UTR: UnTranslated Region
V-ATPase: Vacuolar ATPase
VPS: Vacuolar Protein Sorting

# Summary

Unicellular organisms, as well as multicellular organisms, coordinate growth and division in response to the availability of nutrients. When nutrients are limiting, cells exit the cell cycle in late $G_1$ following completion of the ongoing cell cycle, and enter in $G_0$, a state characterized by a low metabolic activity. Most cells on earth are living in this quiescent state. In contrast, cells that would not properly sense the lack of nutrients usually die. However, in some cases, improper response can lead to uncontrolled proliferation and ultimately to cancer.

The Target Of Rapamycin Complex 1 (TORC1) is a master regulator of cell growth. In higher eukaryotes, TORC1 responds to a wide variety of stimuli (*e.g.* hormones or growth factors), to nutrients as well as to noxious stresses (*e.g.* hypoxia or energy depletion). Among the nutrients, amino acids represent an important class of molecules regulating TORC1 activity and cell growth. In response to amino acids, TORC1 stimulates protein synthesis, transcription, translation initiation, mRNA stability etc., while it inhibits the stress response and degradative processes such as autophagy. Although efforts have been made in deciphering the molecular mechanisms that control TORC1 in response to amino acids, key questions remain unanswered.

The mechanisms of cell growth control by TORC1 are evolutionary conserved in eukaryotes, and the yeast *Saccharomyces cereviasiae* represents a very powerful genetic and biochemical tool to study how cells respond to an amino acid stimulus at the molecular level. We have previously identified in our laboratory a complex of four proteins named the Ego complex (EGOC) as being crucial in relaying an amino acid signal to TORC1. The EGOC is composed of two scaffold proteins Ego1 and Ego3, which allow proper localisation of the complex at the vacuolar rim, and two small Ras-related GTPases Gtr1 and Gtr2 which, when loaded with GTP and GDP, respectively, stimulate TORC1 activity to promote cell growth. It was also demonstrated in higher eukaryotes that an equivalent multiprotein complex plays a similar role, underlying again the conservation of the amino acid signalling pathway, from yeast to man.

The aim of this thesis is to understand how amino acids control the EGOC. The amino acids in a cell are compartmentalized in two pools, a cytoplasmic pool that sustains protein synthesis and a vacuolar pool that serves as a storage reservoir of amino acids for long-term survival. Based on the observation that both the EGOC and TORC1 localise at the vacuolar rim, we investigated the role of the two intracellular pools of amino acids in TORC1 activation. In this context, we made use of chemical and genetic tools to study the role of each pool. In addition, we investigated the role of the vacuolar ATPase (V-ATPase) in TORC1 signalling as this complex enzyme was found to regulate TORC1 in mammals and because previous interactions between the EGOC and the V-ATPase were found earlier in the laboratory. The results, presented in chapter I, indicate that, although vacuolar

amino acids are important for cell growth, they do not seem to participate directly in the activation of TORC1. Moreover, the yeast V-ATPase does not participate in TORC1 signalling.

Furthermore, we examined the role of glutamine in TORC1 signalling. Glutamine was previously found to be an important modulator of TORC1 signalling, but how the level of this particular amino acid control TORC1 is unclear. Glutamine is interesting because nitrogen and carbon assimilation converge on glutamine biosynthesis. The results presented in chapter II confirm the importance of glutamine in TORC1 stimulation; however, its role is independent of the EGOC and the routes by which it acts remain to be elucidated.

Finally, in chapter III, we tried to identify new partners of Gtr1 by using two different screening strategies. We first undertook a yeast two-hybrid screen and, additionally, performed a tandem affinity purification (TAP) followed by mass-spectrometry analysis of the co-precipitating partners. The latter approach allowed us to identify the leucyl-tRNA synthetase Cdc60 as a leucine-dependent interactor of Gtr1 that we confirmed by *in vivo* pull-down experiments. Amino acyl-tRNA synthetases are a class of enzyme, which catalyse the reaction of amino acylation of a cognate tRNA with its specific amino acid. The fidelity of the leucylation of $tRNA^{leu}$ is ensured by the editing or CP1 domain of Cdc60, which harbours a proofreading activity. Interestingly, while investigating the molecular mechanism of leucine signal transduction by Cdc60 using genetic and chemical tools, we found that the editing domain (CP1), rather than the aminoacylation domain, directly interacts with Gtr1 and is required for the control of TORC1 activity. We hypothesize that leucine starvation induces $tRNA^{leu}$ mischarging, which leads to a conformational change of the CP1 domain of Cdc60 and consequent disruption of its interaction with Gtr1, and thus a down-regulation of TORC1 activity. These new findings provide insights into the mechanisms of amino acid sensing and signaling within the conserved TORC1 pathway.

# Résumé

Les organismes unicellulaires, tout comme les organismes multicellulaires, coordonnent la croissance et la division en fonction de la disponibilité en nutriments. Lorsque ces nutriments deviennent limitants, les cellules sortent du cycle cellulaire tardivement dans la phase $G_1$ après avoir accompli le cycle cellulaire en cours, et entre en $G_0$, un état caractérisé par une faible activité métabolique. La plupart des cellules sur terre vivent dans cet état quiescent. À l'opposé, les cellules qui ne détecteraient pas cette carence en nutriments habituellement meurent. Il se peut toutefois qu'une réponse inappropriée conduise à une prolifération non contrôlée et finalement au cancer.

Le complexe Target Of Rapamycin 1 (TORC1) est un régulateur central de la croissance cellulaire. Chez les eucaryotes supérieurs, TORC1 répond à une grande variété de signaux (tels qu'hormones ou facteurs de croissance), aux nutriments ainsi qu'à des stress nuisibles (tels que l'hypoxie ou l'épuisement de l'énergie). Parmi les nutriments, les acides aminés représentent une classe importante de molécules régulant l'activité de TORC1 et la croissance cellulaire. Ainsi, en réponse aux acides aminés, TORC1 stimule la synthèse protéique, la transcription, l'initiation de la traduction, la stabilité des ARNm etc., tandis qu'il inhibe la réponse au stress et les procédés de dégradation tels que l'autophagie. Des efforts ont permis d'appréhender les mécanismes moléculaires contrôlant la croissance cellulaire en réponse aux acides aminés et, bien que notre compréhension de tels phénomènes continue de s'accroître, des questions demeurent.

Les mécanismes du contrôle de la croissance cellulaire par TORC1 sont évolutionnairement conservés chez les eucaryotes, et la levure Saccharomyces cerevisiae représente un outil génétique et biochimique puissant pour étudier la réponse cellulaire au stimulus acide aminé à l'échelle moléculaire . Nous avons identifié précédemment au laboratoire un complexe formé de 4 protéines appelé le complexe Ego comme étant un relais crucial du signal acides aminés vers TORC1. Le complexe Ego est composé de deux protéines de structure Ego1 et Ego3, qui permettent d'ancrer le complexe à la membrane vacuolaire, et de deux petites GTPases apparenté à Ras, Gtr1 et Gtr2 qui, lorsqu'elles sont respectivement chargées avec du GTP et du GDP, stimulent l'activité de TORC1 pour promouvoir la croissance cellulaire. De plus, l'existence d'un tel complexe effectuant un rôle similaire chez les eucaryotes supérieurs est avéré, confirmant la conservation de la voie de signalisation des acides aminés de la levure à l'humain.

L'objectif de cette thèse est de comprendre comment les acides aminés contrôlent le EGOC. Les acides aminés de la cellule sont compartimentés en deux réservoirs, l'un cytoplasmique utilisé pour la synthèse protéique, et un réservoir vacuolaire servant plutôt de réserve pour la survie de longue durée. A partir de l'observation qu'à la fois le EGOC et le TORC1 localise à la membrane vacuolaire, nous avons exploré le rôle de chacun des réservoirs d'acides aminés intracellulaire pour

l'activation de TORC1. Dans ce cadre, nous avons utilisé des outils chimiques et génétiques afin d'étudier le rôle de chaque réservoir. De plus, nous avons déterminé le rôle de l'ATPase vacuolaire (V-ATPase) dans la voie de signalisation TORC1, étant donné que cette enzyme a été proposée comme régulateur de TORC1 chez les mammifères, et que des interactions entre le EGOC et la V-ATPase ont été précédemment mises en évidence au laboratoire. Les résultats, présentés dans le chapitre I, indiquent que, bien que les acides aminés vacuolaires soient importants pour la croissance cellulaire, ils ne semblent participer directement à l'activation de TORC1. De plus, la V-ATPase de levure ne participe pas à la signalisation TORC1.

D'autre part, nous nous sommes attachés à clarifier le rôle de la glutamine dans la voie de signalisation de TORC1. La glutamine a été antérieurement proposée comme un important modérateur de TORC1, mais la façon dont le niveau de glutamine affecte TORC1 demeure floue. La glutamine est intéressante de par sa position à l'interface entre les métabolismes azoté et carboné. Les résultats présentés dans le chapitre II confirment l'importance de la glutamine pour l'activation de TORC1; néanmoins, son rôle semble indépendant du EGOC et les voies par lesquelles elle agit reste à déterminer.

Finalement, dans le chapitre III, nous avons tenté d'identifier de nouveaux partenaires de gtr1 en utilisant deux approches de crible. Nous avons tout d'abord entrepris un crible double-hybride puis, nous avons réalisé une purification d'affinité en tandem (TAP) suivi d'une analyse par spectrométrie de masse des partenaires co-précipitants. Cette dernière approche nous a permis d'identifier la leucyl-ARNt synthetase Cdc60 comme un intéracteur de Gtr1 dont l'association est dépendante de la leucine. Cette interaction a pu être confirmé ultérieurement *in vivo* par co-immunoprécipitation. Les amino acyl-ARNt synthetases sont une classe d'enzymes qui catalyse la réaction d'amino acylation d'un ARNt spécifique avec l'acide aminé correspondant. La fidélité de la leucylation de l'$ARNt^{leu}$ est assurée par le domaine d'éditage (ou CP1) de Cdc60, qui présente une activité de relecture. De manière intéressante, en étudiant les mécanismes moléculaires par lesquels la leucine est signalée à travers Cdc60, et ce à l'aide d'outils génétiques et chimiques, nous avons pu montrer que le domaine CP1, plutôt que le domaine d'aminoacylation, interagit directement avec Gtr1 et, est nécessaire au contrôle de l'activité de TORC1. Nous supposons ainsi qu'une raréfaction de la leucine induit le mauvais chargement des $ARNt^{leu}$, ce qui induit un changement de conformation du domaine CP1 de Cdc60 et, ainsi, la rupture de l'interaction entre Cdc60 et Gtr1. Ces résultats récents offrent un nouvel aperçu de la régulation de la voie de signalisation conservée TORC1.

# - Introduction -

# I. *Saccharomyces cerevisiae* nutritional requirements and control of cell proliferation

Generally, eukaryotic cells must adapt their growth to the availability of nutrients. In yeast, cell growth and division depends mainly on four nutrients, which are carbon, nitrogen, phosphate and sulfur. Limiting amounts of a nutrient may induce a complete reprogramming of the cell's metabolism. Moreover, removal of any single key nutrient blocks cell proliferation and redirects cells to a low metabolic and quiescent state called $G_0$. Adaptation to a changing environment requires nutrient sensing systems and signalling pathways that will trigger the transcriptional and metabolic adjustments.

## 1. Carbon sources

Yeast cells obtain their energy from sugars through a fermentative lifestyle, even in the presence of oxygen, rather than through respiration despite the higher efficiency of the latter one. This fermentative lifestyle, also preferred by tumour cells, gives an advantage to cells as it allows maintaining a fast growth rate. Moreover, among different carbon sources, the fermentable sugars glucose and sucrose are the preferred ones and, upon growth on medium containing glucose, genes required for the utilization of glucose are turned on, while genes required for respiration and utilization of alternative carbon sources are turned off.

Extracellular glucose is sensed by the G-protein coupled receptor (GPCR) Gpr1 and its associated $G\alpha$ and $G\beta$ subunits encoded by *GPA2* and *ASC1*, respectively (Kraakman *et al.*, 1999; Xue *et al.*, 1998; Zeller *et al.*, 2007). Upon glucose binding to Gpr1, Gpa2 associates with the adenylate cyclase Cdc35 to trigger its activity and thus, production of cyclic AMP (cAMP) that will signal further downstream to the protein kinase A (PKA) (Thevelein and de Winde, 1999). In parallel to the GPCR system, the enzymatic activity of Cdc35 is stimulated by the two small GTP-binding proteins Ras1 and Ras2 through direct interaction. Cycling of GTP and GDP in Ras proteins is mediated by two GTPase activating proteins (GAPs), Ira1 and Ira2, and by two guanine nucleotide exchange factors (GEF) Cdc25 and Sdc25. In the presence of glucose, the prevalence of Ras-GTP proteins stimulates Cdc35 activity and consequent accumulation of cAMP activates PKA.

The PKA or cyclic AMP-dependent proteine kinase is highly conserved among eukaryotes, and consists of a heterotetramer composed of two catalytic subunits and two regulatory subunits. The catalytic subunits are redundantly encoded by the *tpk1*, *tpk2* and *tpk3* genes, while only one gene, *bcy1*, encode the regulatory subunit. Bcy1 acts as a pseudosubstrate by binding to the catalytic subunits and restricting their activity. Activation of the catalytic subunits occurs through binding of cAMP to Bcy1, which releases the catalytic subunits (Ptacek *et al.*, 2005; Robertson and Fink, 1998). Following activation, PKA phosphorylates transcription factors, stimulating transcription of genes involved in ribosome biogenesis, cell cycle progression and glycolysis, and represses stress-response

genes (Jorgensen and Tyers, 2004). In addition, PKA positively regulates the activity of enzymes involved in glycogen and trehalose degradation (Uno *et al.*, 1983; Zahringer *et al.*, 2000) and of glycolytic enzymes (Cytrynska *et al.*, 2001; Dihazi *et al.*, 2003; Portela *et al.*, 2006), and negatively controls autophagy (Budovskaya *et al.*, 2005; Yorimitsu *et al.*, 2007). Finally, PKA inhibits entry into quiescence by direct phosphorylation of the Rim15 protein kinase.

The Rgt signalling pathway also responds to glucose via two membrane-spanning sensors, Rgt2 and Snf3. Glucose binding to the receptors relieves repression on hexose transporters genes (Palomino *et al.*, 2006). Synthesis of glucose transporters allows efficient import of glucose to sustain cell growth.

Lastly, the Snf1 kinase complex is activated by the absence of glucose. This complex is homologous to the mammalian AMP-activated protein kinase (AMPK) complex. Glucose control over the activity of the different Snf1 complexes occurs via subcellular localization of the γ-subunits Gal83, Sip1 and Sip2. Snf1 exerts its kinase activity mainly towards transcription factors to control expression of genes required for the metabolism of alternate carbon sources, gluconeogenesis and respiration.

## 2. Phosphate sources

Free phosphate uptake is mediated by the high affinity Pi transporter Pho84 (Bun-Ya Mol Cell Biol 1991)) and the $Na^+$/Pi cotransporter Pho89 (Martinez and Persson, 1998). Phosphate sensing relies on the PHO pathway, which is under the control of intracellular inorganic phosphate (Pi). When intracellular phosphate levels are high, the Pho80-Pho85 complex prevents nuclear translocation of the Pho4 transcription factor. Upon phosphate limitation, the cyclin-dependent kinase inhibitor (CKI) Pho81 inactivates Pho80-Pho85. The resulting dephosphorylation of Pho4 allows its entry into the nucleus where it activates the transcription of genes coding for the secreted acid phosphatases (*PHO5*, *PHO11*, *PHO12*) and the high-affinity phosphate transport system (*PHO84*, *PHO89*), to scavenge for and enhance uptake of $P_i$, respectively (Lenburg and O'Shea, 1996; Oshima, 1997). Additionally, the Pho80-Pho85 complex controls $G_0$ entry by maintaining the Rim15 kinase in the inactive phosphorylated form in the cytoplasm (Wanke *et al.*, 2005).

## 3. Sulfur sources

In yeast, sulfur can be assimilated from both organic and inorganic sources, and is required for the synthesis of sulfur amino acids. The expression of genes involved in sulfur amino acid metabolism is repressed by the high concentration of S-adenosylmethionine, the end product of sulfur amino acid biosynthesis. Upon S-adenosylmethionine limitation, the basic leucine zipper transcription factor Met28, together with the trans-acting factors Cbf1, Met4, Met31 and Met32, binds to

promoters of genes of the sulfur network, and positively regulates their expression to increase sulfur uptake and sulfur amino acid metabolism (Thomas and Surdin-Kerjan, 1997).

## 4. Nitrogen sources

### *i Ammonium*

Ammonium constitutes an excellent source of nitrogen for yeast as it can be readily used for the synthesis of amino acids following its intracellular import. Ammonium uptake occurs by the action of three ammonium permeases, which are Mep1, a medium-affinity high-capacity permease, Mep2, a high-affinity low-capacity permease, and Mep3, a low-affinity high capacity permease. In addition Mep2 plays the role of an ammonium sensor to control pseudohyphal growth of diploid cells. The *MEP* genes are under the control of the nitrogen catabolite repression pathway that will be described later. Following uptake, ammonium is incorporated into glutamate and glutamine (Forsberg and Ljungdahl, 2001)

### *ii Allantoin and Urea*

Allantoin is a product of oxidation of uric acid and purine metabolism. Yeast cells are able to use allantoin as a nitrogen source. Following allantoin uptake by the Dal4 allantoin permease. The molecule is sequentially converted to urea by the action of the *DAL1*, *DAL2* and *DAL3* gene products. Urea is further processed to ammonia and carbon dioxide by the action of the urea amidolyase Dur1,2. Genes in the allantoin degradation pathway are regulated by the nature of the nitrogen sources in the medium and by allantoin itself, or by the intermediate allophanate (Cooper *et al.*, 1980; Magasanik and Kaiser, 2002).

### *iii Amino acids*

Yeast cells can use individual amino acids as nitrogen sources. However, some amino acids are preferred by yeast cells over other amino acids and, often, preferred amino acids repress indirectly the expression of genes required for the assimilation of a non-preferred one (Cooper, 1982). For example, glutamine and asparagine are known to be preferred or good nitrogen sources (Godard *et al.*, 2007; Watson, 1976) as compared to tryptophan, methionine and citrulline, which are described as non-preferred or poor nitrogen sources. Leucine and phenylalanine are of intermediate quality in this respect (Hofman-Bang, 1999; Magasanik and Kaiser, 2002; Watson, 1976) (Table 1).

| Nitrogen source | Generation time (hrs min) |
|---|---|
| Alanine | 2.30 |
| Arginine | 2.25 |
| Asparagine | 2.00 |
| Aspartate | 2.10 |
| Citrulline | 4.30 |
| Glutamine | 2.05 |
| Glutamate | 2.15 |
| Isoleucine | 3.55 |
| Leucine | 3.25 |
| Methionine | 4.05 |
| Ornithine | 4.30 |
| Phenylalanine | 3.20 |
| Proline | 3.15 |
| Serine | 2.15 |
| Threonine | 4.20 |
| Tryptophan | 4.45 |
| Tyrosine | 4.10 |
| Urea | 3.35 |
| Valine | 3.00 |

**Table 1: Generation time of a Σ1278b strain according to the nitrogen source** (Godard *et al.*, 2007)

Cells were grown in minimal buffered (pH=6.1) medium containing 3% glucose and 10 mM of one amino acid except tryptophan at 5mM.

When provided with sufficient carbon, phosphate, sulfur and nitrogen sources, yeast cells synthesise all amino acids that will be incorporated in proteins. The conversion of α-ketoglutarate to glutamate and glutamine by incorporation of one or two ammonia, respectively, and their implication as ammonium donor are central in the amino acid metabolism, and provide a rationale for the preference of yeast cells towards glutamine and glutamate (should they be available) as nitrogen sources. Depending on the biosynthetic pathway, amino acids can be classified into the glutamate family (glutamate, glutamine, arginine, proline and lysine), the pyruvate family (leucine, isoleucine, valine and alanine) the serine family (serine, glycine, cysteine and methionine), the aspartate family (aspartate, asparagine, threonine, cysteine and methionine) and the aromatic family (tryptophan, phenylalanine and tyrosine). The histidine anabolic pathway is a separate entity and is connected to the purine biosynthetic pathway (Ljungdahl and Daignan-Fornier, 2012).

The absence of a preferred nitrogen source activates the nitrogen catabolite repression pathway (NCR) through partially redundant transcription factors to allow expression of genes involved in the metabolism of alternative nitrogen sources such as proline or allantoin (Coffman *et al.*, 1997; Hofman-Bang, 1999; Scherens *et al.*, 2006). These changes in gene expression allow cells to adapt their growth rate to the nitrogen source. Interestingly, preferred nitrogen sources display carbon skeletons that can be readily incorporated in metabolic pathways. For instance, six out of the seven

preferred amino acids yield pyruvate (alanine and serine), α-ketoglutarate (glutamate and glutamine) or oxaloacetate (asparagine and aspartate) following a transamination or a deamination step (Figure 1) (for reviews see (Cooper, 1982; Ljungdahl and Daignan-Fornier, 2012)).

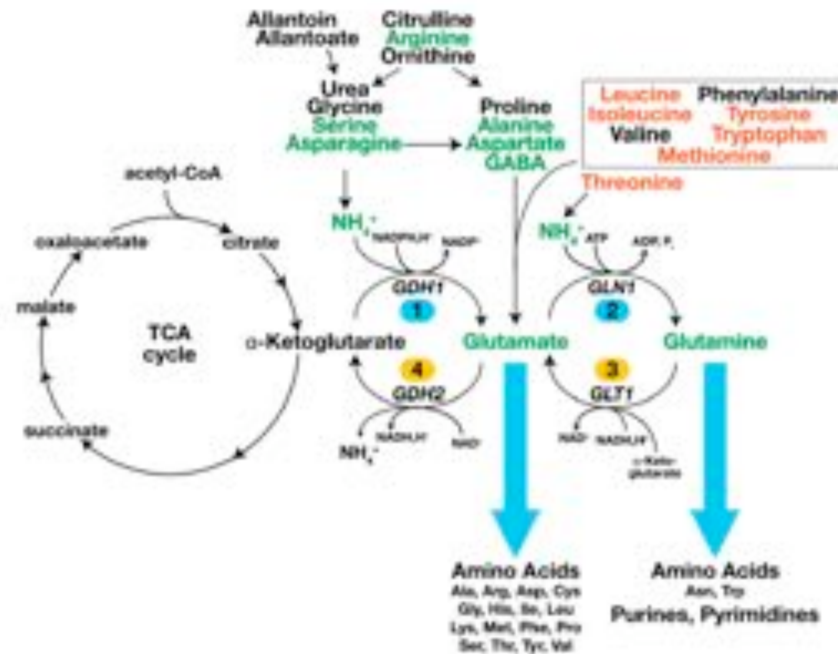



**Figure 1: Schematic of the pathways of nitrogen metabolism (from (Ljungdahl and Daignan-Fornier, 2012))**

The preferred amino acid sources are depicted in green while the nonpreferred are in red. Glutamate and glutamine synthesis depends on the TCA cycle and is governed by two anabolic reactions (1 and 2, blue) catalysed by $NADP^+$-dependent glutamate dehydrogenase (Gdh1) and glutamine synthase (Gln1), and by two catabolic reactions (3 and 4, yellow) catalysed by $NAD^+$-dependent glutamate synthase (Glt1) and $NAD^+$-dependent glutamate dehydrogenase (Gdh2).

During the course of this thesis, we decided to study the role of amino acids in the control of cell growth. Consequently, the following paragraphs will be dedicated to the introduction of amino acid signalling pathways.

# II. Amino acids and signalling pathways

As for the carbon source, yeast cells sense and adapt their growth in response to the amino acid source by employing various sensing and partially redundant signalling pathways. These pathways include (*i*) the SPS signalling pathway, (*ii*) the nitrogen catabolite repression (NCR) pathway, (*iii*) the general amino acid control (GAAC) pathway, (*iv*) the retrograde (RTG) signalling pathway, and (*v*) the EGOC-TORC1 signalling pathway, which will be briefly introduced below (and see Figure 3).

## 1. Amino acids and the SPS pathway

Extracellular amino acids are sensed through the SPS (Ssy1-Ptr3-Ssy5) sensing pathway that works as a ligand-activated receptor to control amino acid permease gene expression. The integral membrane receptor Ssy1 is a unique member of the amino acid permease family as it displays no measurable permease activity (Didion *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999). The capacity of the different amino acids to stimulate Ssy1 differs strongly, with leucine being the most efficient activator (Gaber *et al.*, 2003; Iraqui *et al.*, 1999). Moreover, it was shown that Ssy1 monitors the ratio of external to internal amino acids, and, as such, is sensitive to both signals (Poulsen *et al.*, 2008; Wu *et al.*, 2006). Ssy1 transduces the amino acid signal together with Ptr3 to its partner Ssy5 via an N-terminal extension. Ssy5 is a serine endoprotease composed of a pro-domain and a catalytic domain. Upon amino acid stimulation, the pro-domain is autolytically cleaved and, following phosphorylation by one of the two casein kinases Yck1 and Yck2, is targeted by the SCF Grr1 E2/E3 ubiquitin conjugating system for degradation by the proteasome (Abdel-Sater *et al.*, 2011).

The targets of the remaining catalytic domain of Ssy5 are the two partially redundant transcription factors Stp1 and Stp2, which are expressed as cytoplasmic latent precursors. Stp1 and Stp2 cleavage induces their nuclear translocation where they induce the expression of SPS sensor-regulated genes, together with the transcriptional co-activator Dal81. Stp1 and Stp2 target genes include the amino acid permease genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1*, *MUP1*, *TAT1* and *TAT2*, and the peptide transporter gene *PTR2* (Abdel-Sater *et al.*, 2004; Andreasson and Ljungdahl, 2002; de Boer *et al.*, 2000; Didion *et al.*, 1998; Klasson *et al.*, 1999). In addition, the inner nuclear membrane proteins Asi1, Asi2 and Asi3 prevent access of the leaky unprocessed forms of Stp1 and Stp2 to their target genes (Figure 2) (Boban *et al.*, 2006; Forsberg *et al.*, 2001; Zargari *et al.*, 2007).

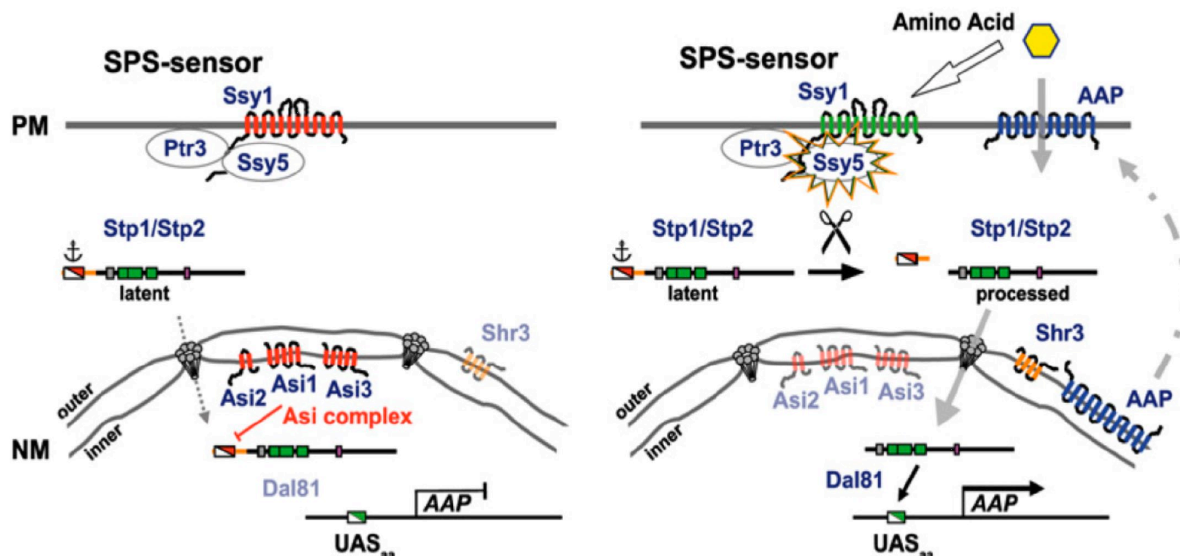



**Figure 2: Schematic of the SPS sensing pathway (from (Ljungdahl and Daignan-Fornier, 2012))**
In the absence of inducing amino acids, Stp1/2 are retained as unprocessed and inactive at the plasma membrane, and expression of amino acid permease (AAP) genes occurs at the basal level (left). In the inner nuclear pore membrane, the Asi proteins prevent leaky Stp1/2 to bind to their target genes.
Upon amino acid stimulation and binding to Ssy1, the Ssy5 protease is activated, and proteolytically processes Stp1/2. The active short forms of Stp1/2 translocate in the nucleus, where, together with Dal81, they bind to the promoter of SPS-regulated genes, especially AAP genes, and activate their transcription. Once they reach the plasma membrane, the AAP increase the uptake of amino acids.

## 2. Amino acids and the nitrogen catabolite repression pathway

A wealth of knowledge indicates that glutamine and glutamate represent key signals controlling NCR gene expression (Beck and Hall, 1999; Blinder *et al.*, 1996; Mitchell and Magasanik, 1984). Expression of NCR genes is regulated by the subcellular localisation of transcriptional activators. On good amino acid sources, Gln3 and Gat1, two GATA transcription factors, localise in the cytoplasm. Gln3 cytoplasmic sequestration is dependent on the protein Ure2, while a yet unidentified protein may anchor Gat1 in the cytoplasm (Coffman *et al.*, 1995; Courchesne and Magasanik, 1988; Magasanik and Kaiser, 2002). Gln3 and Gat1 are phosphoproteins whose phosphorylation status governs their localisation depending on the quality of the nitrogen source. Gln3 gets dephosphorylated upon rapamycin treatment by the Sit4-Tap42 phosphatase module, indicating control of TORC1 over Gln3 (Beck and Hall, 1999; Crespo *et al.*, 2002). However, the link between the Gln3 phosphorylation status and its subcellular distribution remains a matter of debate (Cox *et al.*, 2004). Notably, MSX, an inhibitor of glutamine synthesis, induces, as rapamycin, nuclear translocation of Gln3, although both drugs have opposite effects on Gln3 phosphorylation. In a similar manner, Gat1 regulation is far from being clear and, nitrogen source- or rapamycin-dependent phosphorylation changes have not been demonstrated yet. In addition, it has also been proposed that Gat1 responds to carbon starvation (Kulkarni *et al.*, 2006). The NCR pathway controls, among many other genes, the expression of permeases required for growth on a non-preferred or poor nitrogen source such as the broad-specificity and low-affinity general amino acid permease Gap1 (André *et al.*,

1993; Jauniaux and Grenson, 1990; Van Zeebroeck *et al.*, 2009) or the ammonium permeases Mep1, 2 and 3 (Marini *et al.*, 1997; Marini *et al.*, 1994).

### 3. Amino acids and the GAAC pathway

Upon amino acid starvation, or exposition of cells to unbalanced amino acids, the General Amino Acid Control (GAAC) pathway gets activated (Niederberger *et al.*, 1981). Transcriptional induction of the GAAC is mediated exclusively by the transcription factor Gcn4. Accordingly, upon amino acid starvation, deacetylated tRNAs accumulate in the cytoplasm and bind directly to the histidyl-tRNA synthetase-like domain of the Gcn2 kinase (Dong *et al.*, 2000; Wek *et al.*, 1989). The subsequent conformational change induced by tRNA binding activates Gcn2 which auto-phosphorylates, and then phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2α) (Qiu *et al.*, 2002; Romano *et al.*, 1998). As a result, ternary complex formation is strongly impaired, and the consequent global decrease in translation initiation reduces ribosome scanning and allows translational induction of *GCN4* mRNA after scanning of the four short upstream open reading frames (uORFs) (Abastado *et al.*, 1991; Hinnebusch, 1984; Miller and Hinnebusch, 1989). In addition, Gcn2 is phosphorylated on serine residue 577 in a TORC1-dependent manner and this maintains Gcn2 in an inactive form. Upon amino acid depletion, inactivation of TORC1 activates the Sit4-Tap42 phosphatase module, and subsequent dephosphorylation of S577 activates Gcn2, leading to Gcn4 expression (Cherkasova and Hinnebusch, 2003).This pathway controls the expression of more than 500 genes among which many amino acid and purine biosynthetic enzyme genes and various aminoacyl-tRNA synthetase genes (Delforge *et al.*, 1975; Jia *et al.*, 2000; Mosch *et al.*, 1991; Natarajan *et al.*, 2001; Rolfes and Hinnebusch, 1993).

### 4. Glutamine sensing by the RTG pathway

On glucose medium, the TCA cycle is repressed, and under poor amino acid conditions, yeast cells must maintain the first step of the TCA cycle (*i.e.* from acetyl-CoA to α-ketoglutarate) to sustain amino acid and nucleotide metabolism by providing α-ketoglutarate. Therefore, the TCA cycle functions as a provider of α-ketoglutarate, which is converted to glutamate and glutamine through the successive actions of the glutamate dehydrogenase Gdh1 and the glutamine synthase Gln1. The genes encoding the enzymes required for glutamate synthesis are under the control of the retrograde (RTG) signalling pathway (Liu and Butow, 1999). Thus, under poor ammonium growth conditions, and when mitochondrial functions are reduced (fermentative condition), the RTG pathway serves to ultimately maintain glutamate homeostasis. This pathway consists of four positive regulators (Rtg1, Rtg2, Rtg3 and Grr1) and four negative regulators (Mks1, Bmh1 and Bmh2 and Lst8). Glutamate or glutamine deficiency, as well as mitochondrial dysfunction, directly activates the pathway by targeting the Rtg1-Rtg3 heterodimeric transcriptional activator to the nucleus where it will bind and

activate expression of target genes. Translocation of the complex is regulated by complex interactions among Mks1, Rtg2 and Bmh1/2 (Dilova *et al.*, 2004). Notably, under rich conditions, phosphorylated Mks1 anchors the Rtg1-Rtg3 complex together with Bmh1/2 in the cytoplasm. Upon glutamate/glutamine depletion, Mks1 gets dephosphorylated and, therefore, Rtg2 can compete for binding with Bmh1/2, thus releasing the Rtg1-Rtg3 dimer that enters the nucleus to promote transcription of RTG genes (Dilova *et al.*, 2004). Following release, Mks1 is targeted for degradation by Grr1. Inhibition of TORC1 by rapamycin activates the RTG pathway in a Lst8-dependent manner, although evidence suggests that TORC1 does not participate in nutrient regulation of this pathway (Giannattasio *et al.*, 2005). In this regard, TORC1 control of RTG gene expression may be an indirect consequence of alterations in nitrogen metabolism (Tate and Cooper, 2003).

## 5. Leucine sensing by the EGOC-TORC1 signalling pathway

In yeast, leucine is a very potent activator of the TORC1 signalling pathway, and deprivation of leucine very rapidly downregulates TORC1 activity, while deprivation for histidine or lysine induce a much less rapid decline in TORC1 activity (Binda *et al.*, 2009). Thus, it appears that all amino acids play a role in TORC1 signalling, but leucine seems to play a special predominant role in TORC1 regulation.

The molecular mechanisms by which yeast cells sense amino acids have started to be elucidated with the identification of a multiprotein complex, the EGO complex (EGOC). Mutants in this complex are able to properly enter into $G_0$ following inhibition of TORC1 as shown by acquisition of quiescence specific characteristics. However, *ego* mutants fail to resume growth when cells encounter favorable conditions again (Dubouloz *et al.*, 2005). The name EGO comes from the identification of the members of the complex as playing a role in the Exit from a rapamycin-induced GrOwth-arrest. All three *gtr2*, *ego1* and *ego3* mutants were found in a genetic screen for mutants uncapable of exiting quiescence and re-entering in a proliferating state following a rapamycin-induced TORC1 inactivation (Dubouloz *et al.*, 2005). The Gtr1 GTPase was identified subsequently as a full member of the complex in a study that proposes that EGOC regulates sorting of the general amino acid permease Gap1 (Gao and Kaiser, 2006). Overexpression of Gtr1, Gtr2 or Ego3 proteins confers partial resistance to rapamycin on plate. Additionally, *ego* mutants display cold sensitivity, a defect in the acidification of the vacuole, defects in microautophagy and low TORC1 activity (Binda *et al.*, 2009; Dubouloz *et al.*, 2005; Gao *et al.*, 2005; Nakashima *et al.*, 1996).

Recent studies in yeast, fly and mammals clearly established that, in response to amino acids, EGOC activates TORC1 to promote cell growth (Binda *et al.*, 2009; Kim *et al.*, 2008; Sancak *et al.*, 2008). In 2008, two parallel studies in mammalian cell cultures (HEK293T) and in *Drosophila* have shown that the homologues of Gtr1 and Gtr2, the Rag GTPases (RagA/B and RagC/D, respectively), were acting upstream of TORC1. These studies showed that, in response to amino acids in general, and, more particularly to leucine, the Rag GTPases interact with Raptor, and activate TORC1 to

promote growth. Furthermore, GTP-bound RagA (or Gtr1) and GTP-bound RagC (or Gtr2) are the active signalling forms of the GTPases (Dubouloz *et al.*, 2005; Gao and Kaiser, 2006; Kim *et al.*, 2008; Sancak *et al.*, 2008) and, as such, they can prevent inactivation of TORC1 following amino acid or leucine deprivation by directly interacting with TORC1. In mammalian cells, it was proposed that the Rag GTPases allow proper localisation of TORC1 on the lysosomal membrane where it can be activated by Rheb. Accordingly, the Rag GTPases relay the amino acid signal, while Rheb relays insulin and growth factors inputs (Dennis *et al.*, 2011). High levels of expression of Rheb (around 10 fold the endogenous levels) can bypass the requirement for Rag GTPases in amino acid dependent activation of TORC1, while at levels slightly lower (2.5 fold endogenous levels) Rheb cannot prevent inactivation of TORC1 (Dennis *et al.*, 2011). In yeast, the Rheb homologue Rhb1 does not seem to play a similar function and would rather be implicated in the regulation of arginine uptake (Urano *et al.*, 2000).

In addition to the regulation of TORC1 by the Rag GTPases, it has been shown in yeast that Vam6/Vps39 acts as a Guanine Exchange Factor (GEF) on Gtr1. Vam6 is a member of the HOPS complex and was previously proposed to be the GEF of the Ypt7 GTPase. However, it was lately demonstrated that the Mon1-Ccz1 complex is actually the GEF for Ypt7 (Nordmann *et al.*, 2010). Vam6 localises to the vacuolar rim and, due to its role in the HOPS complex, it regulates the process of tethering during vacuolar fusion. Notably, a *vam6* mutant, that displays fragmented vacuoles, is rapamycin sensitive and presents a low TORC1 activity. This phenotype is not related to the vacuolar morphology defect, as *ypt7* mutants, which have a similar defect in vacuolar morphology appear to have normal TORC1 activity. In addition, over-expression of Vam6 can rescue the growth defect of cells expressing at the same time a WT and a GDP-restricted allele of Gtr1. In addition to these genetic data that place Vam6 upstream of Gtr1 in the TORC1 signalling pathway, it was shown biochemically that Vam6 stimulates GDP release from Gtr1 (Binda *et al.*, 2009). Although, the question of the conservation of Vam6 function in amino acid signalling remains open, a corresponding role was confirmed in a recent study in the fission yeast *S. pombe* (Valbuena *et al.*, 2012).

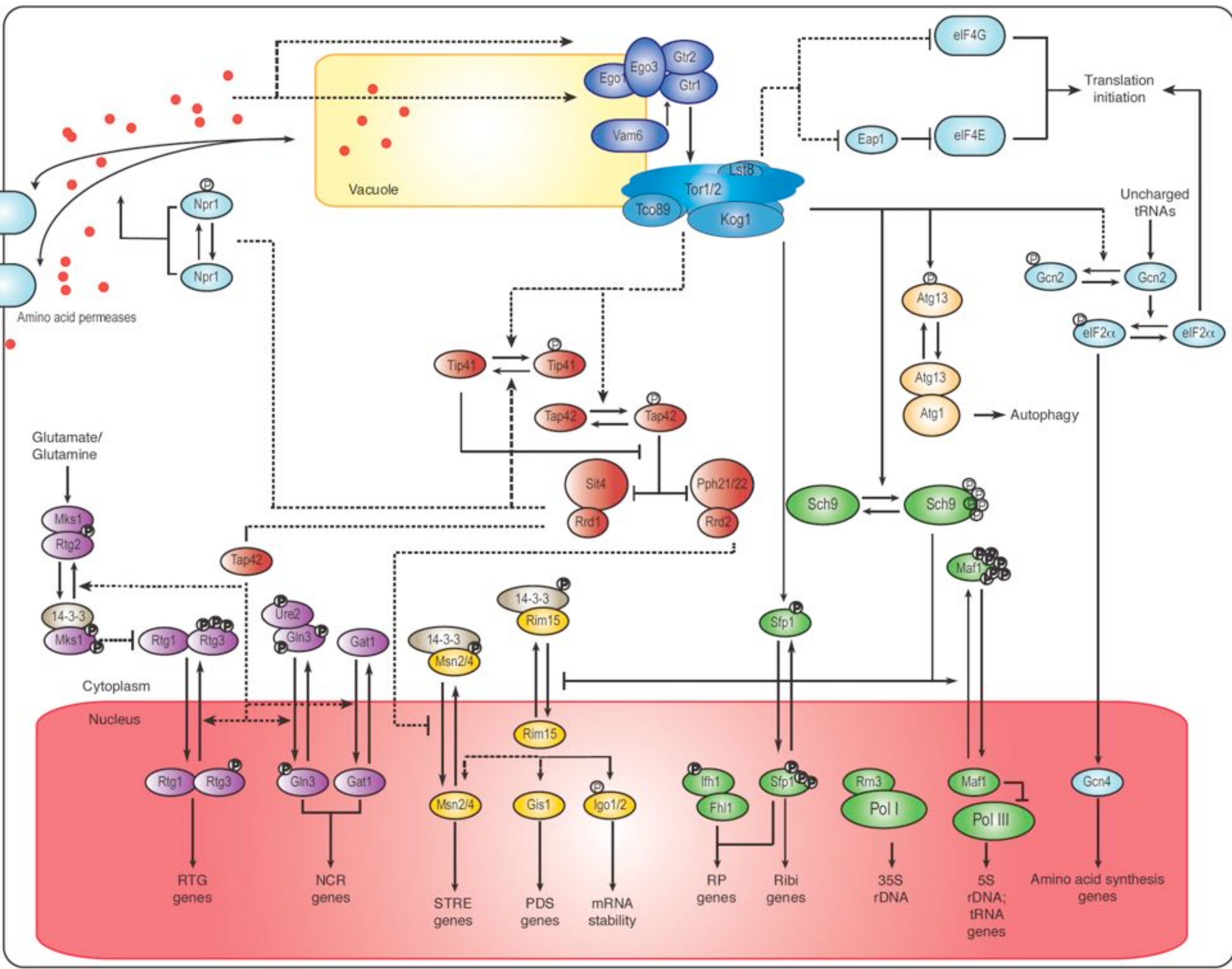
Lately, the three proteins p14, p18 and MP1 that reside on lysosomal membranes, were shown to form a complex that plays a role in mammalian Rag GTPases localisation. This complex was consequently named the Ragulator complex. More precisely, in response to amino acids, the Ragulator complex recruits the Rag GTPases on lysosomal membranes allowing, in turn, the recruitment of mTORC1 through the interaction between the Rag GTPases and Raptor. It has been shown recently that the Ragulator complex is structurally and functionally related to the Ego1 and Ego3 proteins (Kogan *et al.*, 2010).

Some discrepancies between *S. cerevisiae* and HEK293 cells remain. Notably, the study of Sancak showed that Rag GTPases are recruited to lysosomes only upon leucine stimulation while Gtr proteins always localise to the vacuolar rim (Binda *et al.*, 2009; Sancak *et al.*, 2008). However, it has

been reported that mTORC1 localisation is not as strongly affected as it has been proposed, and lysosomal positioning would be changing depending on the nutrient status (Korolchuk *et al.*, 2011). Precisely, under amino acid rich conditions, mTORC1 is associated with peripheral lysosomes placing it close to the plasma membrane and incoming amino acids. Following amino acid starvation, a change in intracellular pH induces mTORC1 relocation to perinuclear lysosomes, in proximity of the site where pre-autophagosomal structures and autophagosome fuse with the lysosome. These data indicate that, under physiological conditions, amino acids govern lysosomes positioning to coordinate anabolic processes and cell growth (Korolchuk *et al.*, 2011).

The EGOC-TORC1 signalling pathway is a central regulator of cells growth in response to amino acid availability. This pathway is highly conserved from yeast to human where it performs the same functions. In addition, many components of this pathway have been linked to tumour development and cancer in mammals. The next chapter will detail the architecture of the two complexes and the downstream processes that they regulate.

**Figure 3: The *S. cerevisiae* amino acids-regulated signalling pathways**
NCR genes expression is regulated by the GATA transcription factors Gln3 and Gat1. The subcellular localisation of the transcription activators depends on the quality of the amino acid source. On poor amino acid sources, Gat1 and Gln3 are targeted to the nucleus where they activate the transcription of genes required for the utilisation of alternative non-preferred nitrogen sources.
The GAAC pathway is activated by the accumulation of uncharged tRNAs which follows amino acids depletion. Deacetylated tRNAs directly bind and activate GCN2 which phosphorylates and inactivates eIF2$\alpha$. The consequent decrease in translation initiation allows induction of *GCN4* mRNA after scanning of the 4 uORFs.
Following glutamine depletion, the Mks1 kinase is dephosphorylated, released from Bmh1/2, and maintained in an inactive state by Rtg2. The phospho-inhibition of Mks1 on the Rtg1-Rtg3 dimer is thus relieved, and the dephosphorylated transcription factors enter the nucleus to trigger the expression of genes required for glutamate synthesis.
TORC1 promotes cell growth by stimulating anabolic processes, notably ribosomes biogenesis and protein synthesis through all three RNA polymerases (green) and, translation initiation and permease activity (light blue). On the other hand, TORC1 inhibition of protein phosphatases (red) represents the main channel of repression of the different stress responses. TORC1 prevents the general stress response mediated by Rim15 and its downstream effectors Msn2/4, Gis1 and Igo1/2 (yellow), and the transcriptional nitrogen response mediated by Rtg1/3, Gln3 and Gat1 (violet). It also inhibits catabolic processes such as autophagy (orange).
Arrows and bars represent positive and negative interactions, respectively. Solid lines symbolise direct interactions, and dashed lines symbolise indirect and/or potential interactions. Circles containing the letter P refer to phosphorylation events.


Ego1
Ego3
Gtr2
Gtr1
Vam6
Vacuole
Lst8
Tor1/2
Tco89
Kog1
eIF4G
Eap1
eIF4E
Translation
initiation
Uncharged
tRNAs
Gcn2
Gcn2
eIF2α
eIF2α
Npr1
Npr1
Amino acid permeases
Tip41
Tip41
Tap42
Tap42
Atg13
Atg13
Atg1
Autophagy
Glutamate/
Glutamine
Mks1
Rtg2
Sit4
Rrd1
Pph21/22
Rrd2
Sch9
Sch9
Tap42
Maf1
14-3-3
Rim15
14-3-3
Mks1
Rtg1
Rtg3
Ure2
Gln3
Gat1
14-3-3
Msn2/4
Sfp1
Cytoplasm
Nucleus
Rim15
Rtg1
Rtg3
Gln3
Gat1
Msn2/4
Gis1
Igo1/2
Ifh1
Fhl1
Sfp1
Rrn3
Pol I
Maf1
Pol III
Gcn4
RTG
genes
NCR
genes
STRE
genes
PDS
genes
mRNA
stability
RP
genes
Ribi
genes
35S
rDNA
5S
rDNA;
tRNA
genes
Amino acid synthesis
genes

# III. EGOC-TORC1 signalling pathway

## 1. Composition of EGOC and TORC1 and functional role of their subunits

### *i The TOR Complex 1 (TORC1)*

#### a. Yeast TORC1

The TORC1 is a multiprotein complex composed of one of the two TOR kinases, Tor1 or Tor2, and the protein Lst8, Kog1 and Tco89 (Chen and Kaiser, 2003; Loewith *et al.*, 2002; Reinke *et al.*, 2004; Wedaman *et al.*, 2003). The complex likely forms a dimer (Wullschleger *et al.*, 2005; Yip *et al.*, 2010) that associates with membranes, and was shown recently to localise predominantly at the vacuolar membrane *in vivo* (Cardenas and Heitman, 1995; Araki *et al.*, 2005; Chen and Kaiser, 2003; Reinke *et al.*, 2004; Sturgill *et al.*, 2008; Wedaman *et al.*, 2003). This later observation is of particular interest regarding the role of amino acid storage within the vacuole.

Tor1 and Tor2 (Target Of Rapamycin 1 and 2) proteins are homologous, share 67% identity and 82% similarity and have both an approximate molecular weight of 281 kDa. The TOR proteins are S/T kinases of the phosphatidylinositol kinase-related protein kinase (PIKK) family and are conserved among eukaryotes, although only fungi harbor two TOR genes. The *TOR2* gene is essential while *TOR1* is not.

TOR is highly structured and contains several domains. The N-terminal part of the protein consists of approximately 20 HEAT (Huntingtin, Elongation factor 3, A subunit of PP2A and TOR) repeats composed of 40 to 50 amino acids forming α-helices and was proposed to mediate protein-protein interactions (Andrade and Bork, 1995; Andrade *et al.*, 2001; Hemmings *et al.*, 1990). This domain is followed by the similar FAT (FRAP, ATM and TRRAP) domain found in all PIKKs, which is accompanied by a FATC domain at the extreme C-terminus. The PI3 kinase domain is located between the FRB (Fpr1-Rapamycin or FKBP12-Rapamycin Binding) and FATC domains.

Both TOR proteins have a largely redundant function although Tor2 performs an additional function inside the rapamycin insensitive TOR Complex 2 (TORC2). The TOR proteins were discovered following a yeast genetic screen to identify the molecular Target Of Rapamycin (Heitman *et al.*, 1991). Rapamycin is a macrolide, produced by a *Streptomyces hygroscopicus* strain isolated from a soil sample from Easter Island or Rapa Nui (Sehgal, 2003), which is now widely used as an antitumor and an immunosuppressant agent. Rapamycin, to inhibit TORC1, needs to associate with the peptidylprolyl isomerase Fpr1, and the rapamycin-Fpr1 complex binds to Tor1 (or Tor2) thus inhibiting specifically the kinase activity of TORC1 (but not TORC2). The composition of the two TOR complexes is summarised in Table 2.

| TORC1 | TORC2 |
|---|---|
| Tor1 or Tor2 | Tor2 |
| Lst8 | Lst8 |
| Kog1 | Avo1 |
| Tco89 | Avo2 |
| | Bit61 |
| | Slm1 |
| | Slm2 |
| | Tsc11 |

**Table 2: Summary of TOR and TOR-associated proteins found in the TOR complexes**

Kog1 (Kontroller Of Growth 1) is an essential protein of 177 kDa and is homologous to mammalian Raptor. Kog1 was identified as a partner of Tor1 by immunopurification of TORC1, and it was shown that depletion or temperature inactivation of Kog1 mimics a TOR deficiency, indicating that Kog1 acts positively in TORC1 (Araki *et al.*, 2005; Loewith *et al.*, 2002). Kog1 contains four HEAT repeats and seven C-terminal WD40 repeats. These protein-protein interaction domains are proposed to act as scaffolds to mediate interaction with TORC1 downstream effectors (Hara *et al.*, 2002).

Lst8 (Lethal with Sec Thirteen 8) is an essential protein of 34 kDa associated with both TORC1 and TORC2, and is homologous to the mammalian Lst8 (mLst8) also known as GβL. The protein was first identified in a screen for mutants which are synthetic lethal with a *sec13* mutant in the secretory pathway (Roberg *et al.*, 1997). Later on, the protein was found associated with the TORC1 components Tor1 and Kog1, and to act positively in TORC1 (Loewith *et al.*, 2002). Lst8 is exclusively composed of seven WD40 repeats and seems to mediate the interaction between TOR and its substrates (Chen and Kaiser, 2003; Liu *et al.*, 2001; Roberg *et al.*, 1997). Unlike yeast Lst8, mLst8 was proposed to relay upstream signals to TORC1 (Kim *et al.*, 2003).

Tco89 (Tor Complex One 89) is a non-essential protein of 89 kDa specific to fungi closely related to *S. cerevisiae*. The protein was first identified following a genetic screen for mutants that block glycerol uptake under osmotic stress (Holst *et al.*, 2000). Loss of *TCO89* renders cells hypersensitive to rapamycin and mimics loss of *TOR1*, suggesting a positive role of Tco89 in TORC1 signalling (Reinke *et al.*, 2004).

b. Mammalian TORC1

Like yeast TORC1, its mammalian counterpart, mTORC1, is composed of the mTOR kinase, Raptor and mLst8, which associate into a complex (Hara *et al.*, 2002; Kim *et al.*, 2002; Loewith *et al.*, 2002). Lately, the Proline-Rich Akt Substrate of 40 kDa (PRAS40) was identified as a new component of mTORC1. PRAS40 interacts with both mTOR and Raptor, and the protein is phosphorylated by mTOR at serine 183, serine 212 and serine 221 (Fonseca *et al.*, 2007; Oshiro *et al.*, 2007; Sancak *et al.*, 2007; Wang *et al.*, 2007; Wang *et al.*, 2008). The exact role of PRAS40 within mTORC1 is unclear and some studies proposed that PRAS40 is inhibitory to the kinase activity of mTORC1 (Sancak *et al.*, 2007; Wang *et al.*, 2007) while others propose a positive role of PRAS40 in mTORC1 activation (Oshiro *et al.*, 2007; Wang *et al.*, 2008). More recently, the DEPDC6 protein was found by co-immunoprecipitation as an interacting partner of mTORC1 (as well as mTORC2) and was consequently renamed DEPTOR. DEPTOR is a 48 kDa protein present only in vertebrates and harbours tandem N-terminal DEP (dishevelled, egl-10, pleckstrin) domains and a C-terminal PDZ (post synaptic density 95, discs large, zonula occludens-1) domain (Peterson *et al.*, 2009), which is required for the interaction with mTOR. Functional analysis of DEPTOR indicates that it acts as an inhibitor of both mTORC1 and mTORC2. Additionally, DEPTOR was found to be overexpressed in many multiple myeloma. The mechanism implies PI3K activation following mTORC1 inhibition in a feedbackloop (Peterson *et al.*, 2009).

As for TORC1, mTORC1 is bound by the FKBP12-rapamycin complex, which inhibits the kinase activity of the complex *in vitro* and *in vivo* (Hara *et al.*, 2002; Kim *et al.*, 2002; Sarbassov *et al.*, 2004). Recently, two studies have started to elucidate the three-dimensional structure of TORC1. The first one, performed on yeast TORC1, details the interaction between TOR and Kog1 by electron microscopy. Due to the high homology of the two yeast TOR proteins, this study could not make a distinction between TOR1 and TOR2, and it was assumed that the TOR proteins adopt a similar structure. This study shows that the TOR N-terminal HEAT repeats form an extended tubular region, already observed in other PIKKs, while the C-terminal domains form a voluminous region. TOR makes extensive interactions with the C-terminal WD40 repeats of Kog1 via its N-terminal HEAT repeats, placing the Kog1 C-terminus in close proximity to TOR kinase domain. This observation would favour a model in which Kog1 recruits TOR substrates (Adami *et al.*, 2007).

The second study, performed on mammalian TORC1, used cryo-electron microscopy on the full complex, and showed that mTOR, mLst8 and Raptor associate stoichiometrically to form a dimer through interlocking interactions between mTOR and Raptor. This was the first confirmation that TORC1 forms effectively dimers and also validated the previous description of TOR structure. Moreover, this work brings further information and more complexity, as it includes Lst8 and maintains the complex in a conformation close to its native conformation. Finally, it provides

evidence of the molecular mechanism by which the FKBP12-rapamycin complex perturbs TORC1 dimerization (Yip *et al.*, 2010).

*ii The Ego complex (EGOC)*

Gtr1 (GTP binding Resemblance protein 1) is a small Ras-related GTPase of approximately 36 kDa and is homologuous to the human RagA and RagB GTPases. Gtr1 localises mainly to the vacuolar membrane but a fraction of it can be found in the nucleus (Bun-Ya *et al.*, 1992). Additionally, Gtr1 was found to be phosphorylated by the Iks1 kinase in a large-scale study (Ptacek *et al.*, 2005), but further data confirming the phosphorylation are missing.

Gtr2 (GTP binding Resemblance protein 2) is a small Ras-related GTPase of approximately 39 kDa homologous to the human RagC and RagD GTPases. Its first description implicated formation of a complex with Gtr1 that was proposed to regulate the Ran/Gsp1 GTPase cycle (Nakashima *et al.*, 1999). As for Gtr1, Gtr2 localises mainly to the vacuolar membrane but a fraction of it can be found in the nucleus (Bun-Ya *et al.*, 1992).

Ego1/Meh1 is a 20 kDa protein localizing to the vacuolar rim, which is highly conserved among fungi, but with no clear homologue in higher eukaryotes. It was first identified as a Multicopy suppressor of Ers1 Hygromycin sensitivy and a *meh1* mutant was shown to be hygromycin sensitive. Ego1/Meh1 encodes a N-terminally myristoylated and palmitoylated hydrophilic protein (Nadolski and Linder, 2009; Roth *et al.*, 2006), which is anchored to the vacuolar membrane. Ego1/Meh1 was shown to recruit Gtr1 to the vacuolar membrane (Gao *et al.*, 2005).

Ego3/Slm4/Nir1 is a 18 kDa protein localizing to the vacuolar rim, which is conserved among fungi, but with no obvious homologue in higher eukaryotes. Its first description came from a synthetic genetic analysis of gene deletions that confer a growth defect when combined with the *cdc42-118* mutant allele, that is conditionally defective in cell polarity establishment (Kozminski *et al.*, 2003). Loss of Slm4 is Synthetic Lethal with loss of Mss4, a Phosphatidylinositol-4-phosphate 5-kinase involved in cytoskeleton organization (Audhya *et al.*, 2004; Desrivieres *et al.*, 1998; Homma *et al.*, 1998).

Ego3, Gtr1 and Gtr2 have been crystallized and the structural studies of the EGOC give insights into assembly of the complex and mechanisms of amino acid signalling. A study of the Ego3 scaffolding protein showed that it forms a homodimer resembling the MP1/p14 heterodimer (figure 4). The structural similarities described in this study confirm the functional conservation of the role of Ego3 and the MP1/p14 dimer in TORC1 signalling. Additionally, even though Ego1 crystal structures are missing, it harbors, like p18, an acid/dileucine vacuolar/lysosomal sorting signal and an N-

terminal myristoylation sequence. This latter sequence allows anchoring at the vacuolar/lysosmal membrane and, as a consequence, Ego1 is thought to serve as a platform for Ego3 dimerization and recruitment of the Gtr proteins. Thus, Ego1/Ego3 likely correspond to the Ragulator complex in higher eukaryotes (Kogan *et al.*, 2010).

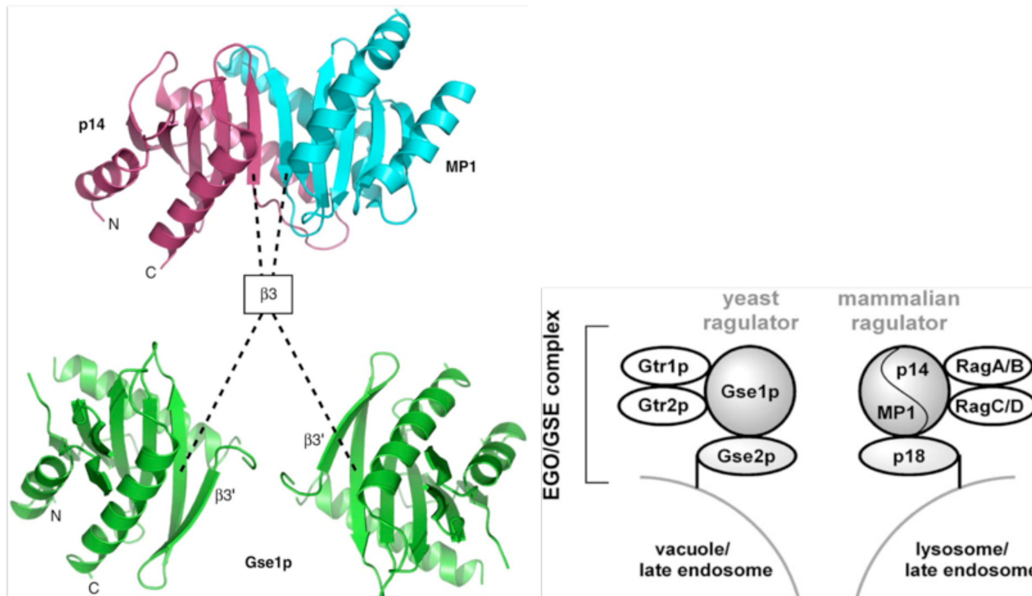



**Figure 4: Three-dimensional structure of Ego3/Gse1 and conservation of the EGO complex (Kogan *et al.*, 2010)**

(A) Ribbon representation of the overall structure of the Ego3 protein with the β3' strand implied in dimerization as compared to the p14/MP1 heterodimer.

(B) Proposed model of the EGO/GSE complex and its mammalian equivalent.

The structure-function study of Ego1 and Ego3 gives insights into the mechanism of membrane anchoring of the whole EGO complex. On the other hand, the Gtr1/Gtr2 heterodimer arrangement shows how the GTPases can possibly interact with and activate TORC1. Gtr1/Gtr2 can be separated into two domains, an N-terminal GTPase (G) domain responsible for GTP binding and hydrolysis, and a C-terminal domain (CTD) mediating the dimerization of the proteins through extensive interactions (Figure 5).

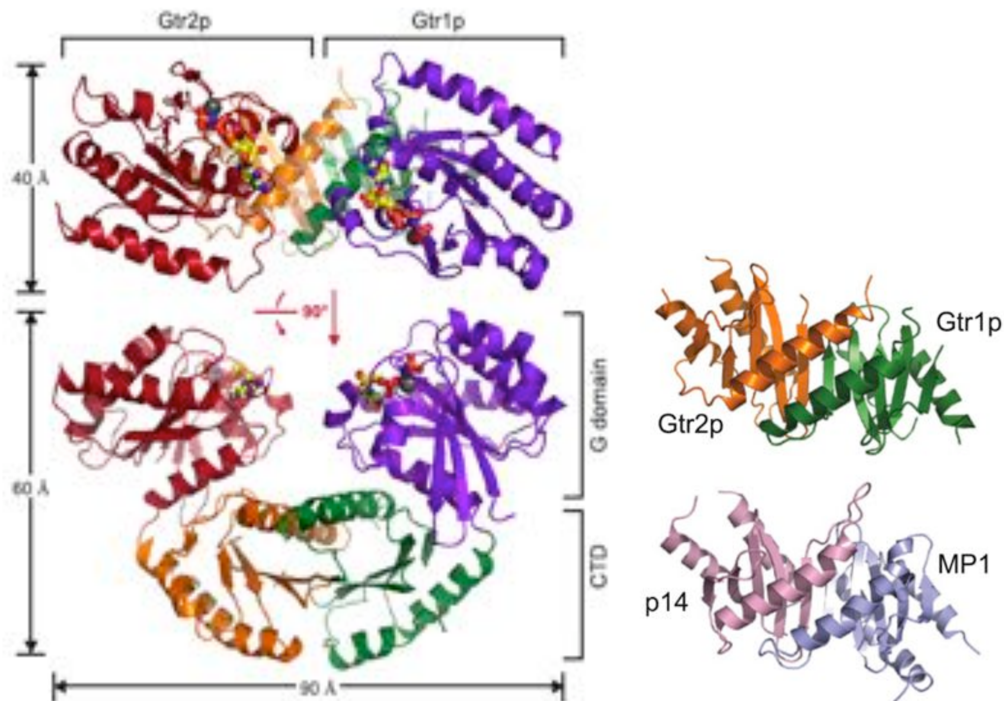



**Figure 5: Three-dimensional structure of the Gtr1-Gtr2 heterodimer (Gong et al., 2011)**
(A) Ribbon representation of the overall structure of the Gtr1-Gtr2 dimer in two different views.
(B) Ribbon representation of the C-terminal domains of the Gtr1-Gtr2 dimer as compared to the p14/MP1 heterodimer.

The two G domains are facing each other with their corresponding surfaces oriented in opposite directions. Inside each monomer, the G domain also makes interactions with the CTD although the nucleotide-binding pocket remains far from the CTD. The overall conformation of the complex might not be affected by nucleotide binding but, upon nucleotide exchange, the switch regions might undergo conformational changes to allow Kog1 recognition and TORC1 activation. Additionally, Gtr1 preferentially in its GTP-bound state recruits Kog1 while the Gtr2 loading status modestly affects this interaction, indicating a preeminent role of Gtr1 in TORC1 activation, which is in line with previous observations (Binda *et al.*, 2009). Lastly, it has been shown that the CTD of the dimer presents a similar three-dimensional structure to the p14/MP1 dimer although they do not share sequence similarity (Gong *et al.*, 2011).

## 2. TORC1 downstream effectors

Although many cellular processes are under the control of TORC1, the underlying molecular mechanisms remains poorly defined, and only two effectors that account for the majority of cell growth control by TORC1 have been well characterized: the protein kinase Sch9 and the type 2A and type 2A-like phosphatases.

*i PP2A and PP2A-related phosphatases*

TORC1 control of phosphatases occurs via phosphorylation of the cytoplasmic anchor protein, Tap42. In its phosphorylated form, Tap42 interacts with the catalytic subunits of the type 2A protein phosphatase (PP2A) Pph21/22, in a Tap42-Rrd2-Pph21/22 complex, or with the related PP2A-like phosphatase Sit4, in a Tap42-Rrd1-Sit4 complex (Di Como and Arndt, 1996; Jiang and Broach, 1999; Zheng and Jiang, 2005). Following TORC1 inactivation, the Tap42-phosphatase complex is liberated into the cytoplasm where Tap42 decays to the dephosphorylated form and dissociates from the catalytic subunits (Yan *et al.*, 2006). The fact that Sit4 and Tap42 are both required for dephosphorylation of some TORC1 downstream targets indicates that Tap42 is a positive regulator of Sit4 phosphatase activity, notably towards Gln3 and Rtg1-Rtg3. Furthermore, Tap42 might dictate the specificity for the phosphatase towards certain substrates (Duvel *et al.*, 2003). Another partner of PP2A and PP2A-like phosphatases is the Tip41 protein, identified by two-hybrid as an interactor of Tap42 (Jacinto *et al.*, 2001). Tip41 is phosphorylated in a TORC1-dependent manner, and in concert with Tap42 redirects the phosphatases activities in response to TORC1 activity (Duvel *et al.*, 2003; Van Hoof *et al.*, 2005; Zheng and Jiang, 2005).

*ii Sch9*

As described previously, Sch9 is a major regulator of ribosome biogenesis and consequently of protein synthesis. Sch9 is a non-essential protein kinase of the AGC family (named according to its members PKA, PKG and PKC) of protein kinases (Pearce *et al.*, 2010). Because of its homology with the mammalian PKB, Sch9 was initially proposed to be its yeast equivalent (Fabrizio *et al.*, 2001; Jorgensen *et al.*, 2002), but this hypothesis was later invalidated following a study that established that Sch9 is rather the yeast ortholog of the mammalian S6 kinase (S6K) and that, like its mammalian counterpart, is phosphorylated by TORC1 (Urban *et al.*, 2007). Activation of Sch9, like for any AGC kinases, requires phosphorylation of the activation loop in the catalytic domain, together with phosphorylation of an hydrophobic motif located at the C-terminus of the protein. Further phosphorylation of the turn motif (TM) located between the hydrophobic motif (HM) and the kinase domain stabilizes the protein. TORC1 phosphorylation on Sch9 occurs on six serine/threonine residues that lie in the HM motif of the protein. Additionally, Sch9 is a target of Pkh1 and Pkh2, which phosphorylate the kinase at a residue in the activation loop, which, together with TORC1-mediated phosphorylations of the TM and HM motifs, are required for full activation of Sch9 (Urban *et al.*, 2007).

## 3. Processes under the control of TORC1 effectors

Starvation for amino acids as well as exposure to rapamycin induces a cell cycle arrest in G1 and transition into quiescence. It is now well established that TORC1 transmits an amino acid signal to downstream effectors to promote cell growth, i.e. accumulation of mass. Particularly, TORC1 positively regulates cell growth while it negatively controls stress responses. In the following paragraphs I will focus on some general aspects of cell growth control by TORC1 and its effectors.

### *i Transcriptional control*

In exponentially growing cells, the production of ribosomes accounts for more than 90% of the total transcription and reaches approximately 2000 ribosomes per minutes, which consumes an important part of the cell's resources and energy. The trancription of rRNA genes by the DNA-dependent RNA polymerase I (Pol I) represents 60% of the total transcription, and 50% of Pol II transcription is dedicated to the transcription of the 137 ribosomal proteins (RPs) genes and more than 200 ribosome biogenesis (Ribi) factor genes (Warner, 1999). As a consequence, the first level of regulation of protein synthesis occurs at the transcriptional level and, following TORC1 inhibition, the expression of rRNA, RP and Ribi genes is drastically decreased (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Powers and Walter, 1999; Zaragoza *et al.*, 1998). It has been shown in mammalian cells that association between the transcriptional coactivator TIF-1A and the Pol I subunit RPA43 is directly controlled by mTORC1 phosphorylation and this may also hold true in yeast (Laferte *et al.*, 2006; Mayer *et al.*, 2004). An interesting observation also reports that rRNA production by Pol I may regulate the activity of the two other RNA Polymerases. Indeed, a fusion between the Pol I transcriptional activator Rrn3 and the Rpa43 Pol I subunit leads to constitutive activation of Pol I, but also to a deregulation of Pol II and Pol III, specifically with respect to RP and Ribi genes transcription, and 5S rRNA, respectively (Chedin *et al.*, 2007; Laferte *et al.*, 2006). However, the mechanism behind this phenomenon is unclear, but might imply rRNA processing factors (Rudra *et al.*, 2007). Moreover, several Pol II transcription factors regulating RP gene expression are under the control of the Sch9 branch of the TORC1 signalling pathway (Huber *et al.*, 2009).

A key transcription factor for RP gene expression is Fhl1 (Forkhead-like 1), which recruits the Ifh1 coactivator in a nutrient-dependent manner (Martin *et al.*, 2004; Rudra *et al.*, 2007; Schawalder *et al.*, 2004; Wade *et al.*, 2004). The split $Zn^{2+}$-finger transcription factor Sfp1 drives expression of a subset of RP genes and of a large set of Ribi genes following TORC1 phosphorylation (Lempiainen *et al.*, 2009). TORC1 regulation of ribosome biogenesis occurs as well on Pol III, which transcribes 5S rRNA and tRNAs, and is probably the best described regulation. The highly conserved repressor of Pol III transcription, Maf1, is under the direct control of the TORC1 effector kinase Sch9, whose phosphorylation retains Maf1 in the cytoplasm, thus preventing repression of Pol III (Huber *et al.*, 2009; Lee *et al.*, 2009; Pluta *et al.*, 2001; Upadhya *et al.*, 2002).

In stationary phase, following TORC1 inactivation, general transcription is shut down. However, a genome wide study of RNA Pol II location during transition from exponential growth to stationary phase showed that 460 transcripts are enriched, most of them being induced during the diauxic shift. These transcripts include stress-response genes and genes required for the adaptation to a limited nutrient source. It has also been observed that Pol II, although inactive, is still present on promoters in quiescent cells. In addition, it has been proposed that quiescent cells may still be responsive to environmental changes without leaving quiescence (Radonjic *et al.*, 2005).

The transcriptional reprogramming required for the entry into quiescence is under the control of the PAS kinase Rim15. In exponential phase, the TORC1 downstream effector Sch9 directly phosphorylates Rim15 on a specific serine/threonine residue, thus promoting Rim15 association with the 14-3-3 protein Bmh2, and subsequent cytoplasmic retention. Additionally, TORC1 inhibition of PP2A phosphatases prevents Rim15 dephosphorylation and nuclear import (Pedruzzi *et al.*, 2003; Wanke *et al.*, 2005). Following TORC1 inactivation, activated Rim15 drives the transcriptional reprogramming through its positive effect on the general stress transcription factors Msn2/Msn4 and the post-diauxic shift transcription factor Gis1. The precise mechanism by which Rim15 controls these transcription factors is not known, but it could modulate their activity and/or their nuclear localization (Cameroni *et al.*, 2004; Pedruzzi *et al.*, 2000; Wei *et al.*, 2008).

*ii Stability of the mRNAs*

Following transcription, the control of the stability of mRNAs is an important aspect of gene expression and, although TORC1 has been implicated in mRNA stability control, the precise mechanism is still unknown. Upon amino acid depletion, translation initiation is progressively decreased together with ribosomal protein and translation factor gene expression (Boucherie, 1985; DeRisi *et al.*, 1997; Ju and Warner, 1994). These changes result in a rapid decrease in general protein synthesis required for the sake of energy sparing. However, as discussed above, a fraction of genes likely involved in different aspects of stress tolerance and maintenance of viability are induced, and the corresponding mRNAs need to be protected from degradation (Radonjic *et al.*, 2005). The fate of the mRNA depends on structural properties within the 5’- and 3’-untranslated regions (UTR) of the mRNA, sequence specificities within the 3’-UTR, and association with translational repressors. Recently, it has been shown that following TORC1 inhibition, activation of the Rim15 kinase activates the paralogous Igo1 and Igo2 proteins, which protect mRNAs from degradation (Talarek *et al.*, 2010).

## 4. TORC1 control of amino acid homeostasis

As described previously, amino acids are the main regulators of TORC1. However, in response to amino acids, TORC1 also modulates various cellular processes, which are tightly linked to intracellular amino acid homeostasis. This response, which allows a proper adaptation of cell growth to the environmental as well as to the intracellular amino acid availability, will be introduced in the section below.

### *i Regulation of amino acid permeases*

*S. cerevisiae* encodes 19 amino acid transporters whose expression is regulated. Under nutrient rich conditions, high-affinity and high-selectivity permeases are expressed and targeted to the plasma smembrane. Upon amino acid deprivation, these transporters are internalized and targeted for degradation in the vacuole whereas a few low-affinity, high capacity and broad-specifity permeases are expressed and targeted to the plasma membrane. It is now clearly established that the sorting of the high-affinity and low-affinity transporters is respectively negatively and positively regulated by the protein kinase Npr1, whose activity is itself repressed by TORC1 (Schmidt *et al.*, 1998). Thus, TORC1 inactivation relieves the phospho-inhibition on Npr1, which, in turn, phosphorylates the α-arrestin Aly2 to redirect the broad-specificity permease Gap1 to the plasma membrane by a yet to be defined mechanism (O'Donnell *et al.*, 2010). Furthermore, Npr1 phosphorylation of different ubiquitin ligase adaptors induces their relocalisation to the ER, and allows stabilisation at the plasma membrane of low-affinity permeases normally targeted by these ubiquitin ligase adaptors (De Craene *et al.*, 2001; MacGurn *et al.*, 2011).

### *ii Control of translation initiation*

TORC1 positively modulates translation initiation by regulating the phosphorylation status and activity of Gcn2, as described earlier. In addition, TORC1 promotes translation initiation by negatively regulating the eIF4E-associated protein Eap1 and stabilising the eIF4G protein. It has been reported that rapamycin induces a rapid degradation of eIF4G. The eIF4G protein is part of the eIF4F complex (eIF4E-eIF4G-eIF4A) required for recognition of the 5'-cap structure on the mRNA and subsequent ribosome binding (Barbet *et al.*, 1996; Cosentino *et al.*, 2000). Furthermore, TORC1 represses the activity of Eap1, a competitor of eIF4G in the binding of eIF4E, thus promoting cap-dependent translation initiation (Berset *et al.*, 1998). Of note, although evidence of TORC1 regulation on Eap1 is missing, the mammalian homologue of Eap1, 4E-BP1, is directly phosphorylated by mTORC1 (Burnett *et al.*, 1998). Through this mechanism, TORC1 is thought to drive cell cycle progression, as the $G_1$ cyclin Cln3 depends on cap-dependent protein synthesis. Thus, upon TORC1 inhibition, Cln3 levels decrease, which explains in part the corresponding $G_1$ cell cycle arrest in

rapamycin-treated cells (Barbet *et al.*, 1996; Gallego *et al.*, 1997). In addition, a decreased rate of translation initiation maintains the level of free cytoplasmic amino acids.

*iii Regulation of autophagy*

Autophagy is a conserved process in eukaryotes where bulk cytoplasm and organelles wrapped in a double lipid bilayer, the autophagosome, are transported to the lysosome for degradation. This phenomenon is induced upon starvation for any nutrients (carbon, amino acids, nitrogen, phosphate, sulfur) or upon ER stress and is characterized by the formation of a double-membrane vesicles in the proximity of the lysosome/vacuole at a site called the pre-autophagosomal structure (PAS). Currently, 31 autophagy-related genes (*ATG*) have been idientified in *S. cerevisiae* that can be classified into 3 groups relative to their function: non selective autophagy or macroautophagy (20 genes), selective autophagy (9 genes) and degradation/recycling of autophagosomes (2 genes) (Nakatogawa *et al.*, 2009; Wang and Klionsky, 2003).

The proteins involved in non-selective autophagy are required for the formation of the phagophore/pre-autophagosome. Except the proteins of the Atg1 kinase complex, all components are shared by the cytoplasm to vacuole (Cvt) pathway. The formation of PAS is a two-step mechanism composed of a nucleation step where a minimal-size autophagosome forms, and an expansion step where the membrane extends (Abeliovich *et al.*, 2000). Deletion of any of the genes implicated in the nucleation process prevents macroautophagy and impairs survival upon nutrient starvation.

Selective autophagy is the process of autophagy directed towards organelles, misfolded proteins or protein aggregates and pathogens, and implicates recognition of the substrate to be targeted for degradation by specific adaptor proteins. Such processes have been described and renamed depending on the substrate, *e.g.* mitophagy for mitochondria (Kissova *et al.*, 2004), pexophagy for peroxisomes (Sakai *et al.*, 1998), ribophagy for ribosomes (Kraft *et al.*, 2008), xenophagy for pathogens (Dupont *et al.*, 2010) and aggregaphagy for protein aggregates (Webb *et al.*, 2003).

Autophagy is a tightly regulated process. In *S. cerevisiae*, under rich conditions, the concerted action of the TORC1 and Ras/PKA pathways prevents induction of autophagy by different means. TORC1 phosphorylates the Atg1 kinase partner Atg13, preventing interaction between the two proteins and activation of Atg1 (Kamada *et al.*, 2000; Kamada *et al.*, 2010). On the other hand, PKA-mediated phosphorylation of Atg1 and Atg13 prevents their interaction with other autophagy components, thus preventing PAS formation (Figure 6) (Stephan *et al.*, 2009). Upon prolonged starvation, it is proposed that amino acids recycled from the vacuole could partially reactivate TORC1 and ultimately attenuate autophagy (Shin and Huh, 2011). Although the regulation of Atg1 activity is a conserved feature from yeast to mammals, slight differences exist in the regulation of Atg1 complex formation, which are discussed by Chen and Klionsky (Chen and Klionsky, 2011).

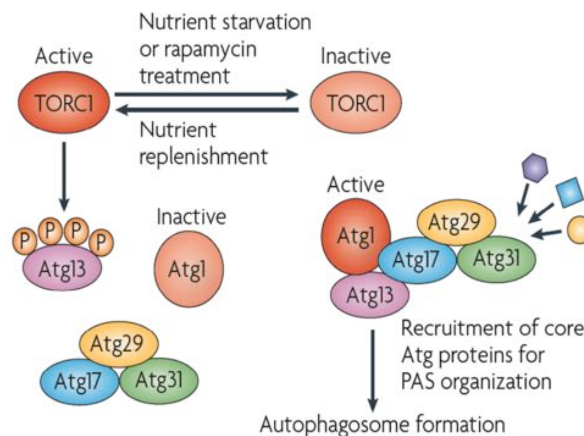



**Figure 6: Regulation of autophagosome formation by TORC1 (Nakatogawa *et al.*, 2009)**
Following TORC1 inactivation, Atg13 is dephosphorylated, promoting its association with and activation of Atg1 kinase, and thus, formation of the PAS.

Once the autophagosome reaches the vacuolar membrane, it fuses with the vacuole using the conventional fusion machinery (Darsow *et al.*, 1997; Fischer von Mollard and Stevens, 1999; Sato *et al.*, 1998) and releases a single membrane autophagic body inside the vacuolar lumen. Finally, the protein lipase Atg15 digests the membrane of the autophagosome making its content accessible to vacuolar hydrolases (Epple *et al.*, 2001; Teter *et al.*, 2001). Amino acids resulting from this degradation are recycled back into the cytosol thanks to the integral vacuolar membrane effluxer Atg22 together with additional amino acid vacuolar transporters (Yang *et al.*, 2006). The role of the vacuole in amino acid homeostasis will consequently be introduced in the next section.

# IV. The yeast vacuole

The yeast vacuole is the storage compartment for metabolically important compounds such as amino acids, polyphosphates, $Ca^{2+}$ and ions. These stores are particularly relevant for cells to survive upon transient deprivation, for buffering cytosolic levels, or for revival after prolonged periods of nutrient scarcity.

Previous reports proposed that the pool of vacuolar amino acids may play a role in the control of TORC1 (Dubouloz *et al.*, 2005; Zoncu *et al.*, 2011). In addition, some vacuolar amino acid transporters are phosphorylated in a TORC1 dependent-manner (Huber *et al.*, 2009), but the consequences of such modifications on the transporters activities remains unknown. Interestingly, mutants defective in vacuolar morphology show a synthetic growth defect or synthetic lethality with a *tor1* mutant. Furthermore, the corresponding synthetic lethality can be suppressed by addition of glutamate or glutamine to the medium, indicating that amino acid metabolism and vacuolar functions are intertwined. This was notably shown for class C-VPS components (Zurita-Martinez *et al.*, 2007). Class C-VPS mutants have no visible vacuole and consequently low levels of intracellular amino acids, and are particularly sensitive to amino acid starvation (Bowers and Stevens, 2005; Zurita-Martinez *et al.*, 2007). In addition, both EGOC and TORC1 were found to localise at the vacuolar rim, which places again the vacuolar amino acids as potential modulators of TORC1 activity (Binda *et al.*, 2009; Sturgill *et al.*, 2008). Taken together, the vacuole plays an important role in amino acid metabolism, and I will therefore round up this introductory chapter with a brief, but more detailed, description of the yeast vacuole.

## 1. Vacuolar amino acid transport

The major pool of uptaken amino acids is not involved in metabolic processes but rather inert (Davis, 1972; Oaks and Bidwell, 1970) and, as a consequence, this inactive pool needs to be stored in a separate compartment, the vacuole. Vacuolar amino acids, by acting as nitrogen suppliers, contribute to the homeostasis of the cytoplasmic pool of amino acids that take part in the metabolism of the cell.

The main amino acids stored in the vacuole are the basic (arginine and lysine essentially) and neutral amino acids (asparagine and glutamine), indicative of a selective uptake for a subset of amino acids. These amino acids usually have a low metabolic turnover, and arginine, the amino acid with the highest nitrogen content, accounts for the largest reserve of nitrogen (Kitamoto *et al.*, 1988). Amino acid import into the vacuole is ensured by transport systems in the vacuolar membrane. Transporters of the major facilitator superfamily (MFS) carry out the uptake and export of basic amino acids while large neutral amino acids are transported by $H^{+}$/amino acid antiport systems which belong to the amino acid/auxin permease (AAAP) family (Saier, 2000) called the amino acid vacuolar transporter

(AVT) family in yeast (Russnak *et al.*, 2001). Notably, Avt1 is responsible for the uptake of glutamine, leucine, isoleucine, asparagine, and tyrosine while the transporters Avt3 and Avt4 counterbalance the action of Avt1. Although the proteins involved in the uptake of glutamate and aspartate are not known, Avt6 exports those amino acids from the vacuole to the cytoplasm. The substrates of the remaining members of this family of transporter at the vacuolar membrane have not been uncovered yet (for details see Figure 7 and Table 3 adapted from (Sekito *et al.*, 2008)).

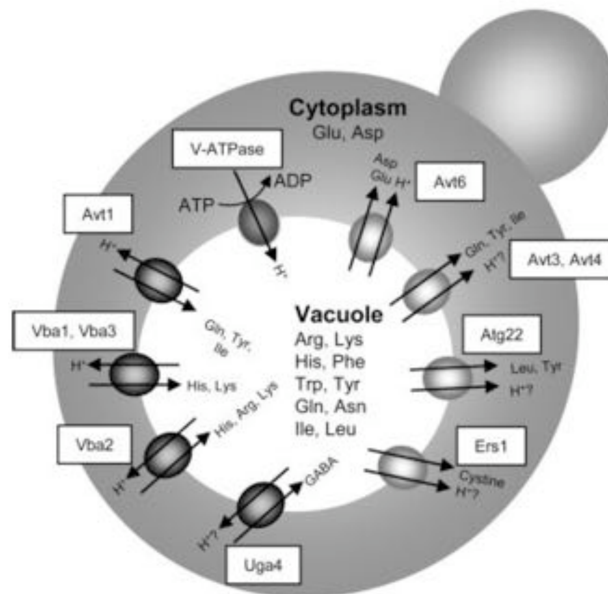



**Figure 7: Diagram of the amino acids import and export at the vacuolar membrane (from (Sekito *et al.*, 2008)**

The vacuole stores ten amino acids (Arg, Lys, His, Phe, Trp, Tyr, Gln, Asn, Ile and Leu), predominantly basic amino acids (Arg, His, Lys), while acidic amino acids are completely excluded. Most of the amino acid transporters have been identified also their specificity remains to be fully determined. Most of these transporters couple amino acid import with the proton gradient generated by the $H^+$ V-ATPase.

On the other hand, Vacuolar Basic Amino acid (VBA) transporters are involved in the uptake of arginine, lysine and histidine (Shimazu *et al.*, 2005). In particular Vba1 and Vba3 ensure the transport of histidine and lysine and Vba2 additionally imports arginine.

Apart from these two main families of vacuolar amino acids transporters, additional transporters have been suggested to mediate amino acid transport. For example, Uga4 is a potential candidate for the import of GABA inside the vacuole (Uemura *et al.*, 2004). Atg22, a protein belonging to the autophagy-related family of proteins, plays a crucial role in amino acid export from the vacuole following autophagy (Yang *et al.*, 2006). Ers1, a homologue of the human cystinosin protein might be involved in the export of cystine although evidence for such an activity is still lacking (Gao *et al.*, 2005).

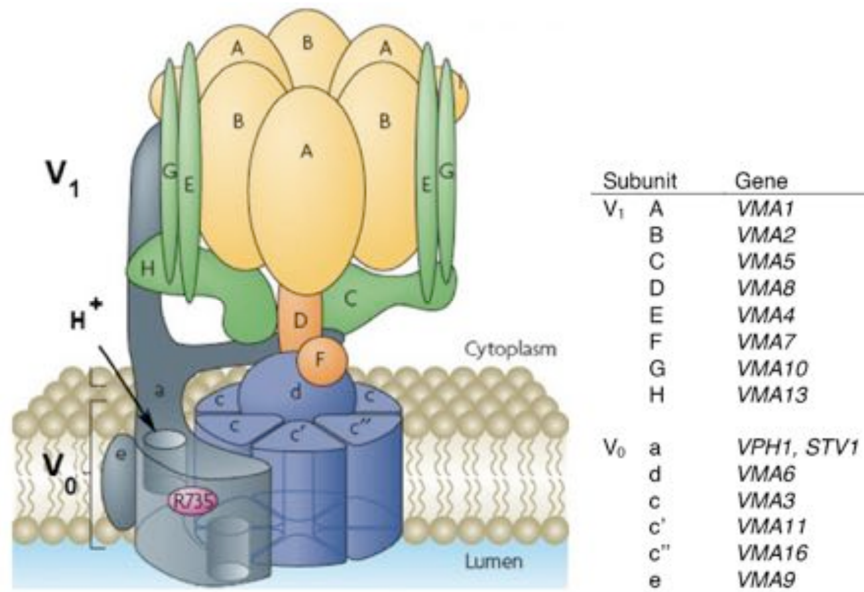
| Family | Subfamily | Protein | Selectivity | Vacuolar direction | Reference |
|---|---|---|---|---|---|
| AAAP | AVT | Avt1 | Gln. Asn, Ile, Leu, Tyr | In | (Russnak *et al.*, 2001) |
| | | Avt2 | Unknown | | (Russnak *et al.*, 2001) |
| | | Avt3 | Gln. Asn, Ile, Leu, Tyr | Out | (Russnak *et al.*, 2001; Yang *et al.*, 2006) |
| | | Avt4 | Gln. Asn, Ile, Leu, Tyr | Out | (Russnak *et al.*, 2001; Yang *et al.*, 2006) |
| | | Avt5 | Unknown | | (Russnak *et al.*, 2001) |
| | | Avt6 | Asp, Glu | Out | (Russnak *et al.*, 2001) |
| | | Avt7 | Unknown | | (Russnak *et al.*, 2001) |
| MFS | VBA | Vba1 | His, Lys | In | (Shimazu *et al.*, 2005) |
| | | Vba2 | His, Arg, Lys | In | (Shimazu *et al.*, 2005) |
| | | Vba3 | His, Lys | In | (Shimazu *et al.*, 2005) |
| | | Vba4 | Unknown | | (Shimazu *et al.*, 2005) |
| | | Vba5 | Unknown | | (Shimazu *et al.*, 2005) |
| | | Atg22 | Leu, Tyr, Ile | Out | (Yang *et al.*, 2006) |
| APC | | Uga4 | GABA | In | (Uemura *et al.*, 2004) |
| LCT | | Ers1 | Cystine | Out | (Gao *et al.*, 2005) |

**Table 3: *Saccharomyces cerevisiae* vacuolar amino acids transporters** (adapted from (Sekito *et al.*, 2008)

## 2. Vacuolar amino acids and pH

Acidification of the vacuole is essential to the vacuole as the proton gradient is required for the uptake of amino acids, and the acidic pH offers optimal conditions for the maturation and catalytic activity of hydrolases that reside in the lumen of the vacuole.

The vacuolar pH varies between 4.6 and 5.0 depending on the growth conditions. As a consequence, the vacuole represents the most acidic compartment of the cell and almost all of its functions are tied to its pH (Mellman *et al.*, 1986). Acidification is enabled due to the vacuolar $H^+$-ATPase (V-ATPase), a proton pump that couples ATP hydrolysis to proton transport from the cytosol to the lumen of the vacuole. The V-ATPase is composed of 8 peripheral subunits (A to H) forming the $V_1$ domain and 6 integral membrane subunits (a, c, c', c'', d and e) forming the $V_0$ domain (Figure xx). The $V_1$ domain is responsible for ATP hydrolysis while the $V_0$ domain transports protons across the membrane. The V-ATPase is closely related to the mitochondrial F-ATPase and functions similarly (Figure 8).




| Subunit | | Gene |
|---|---|---|
| $V_1$ | A | *VMA1* |
| | B | *VMA2* |
| | C | *VMA5* |
| | D | *VMA8* |
| | E | *VMA4* |
| | F | *VMA7* |
| | G | *VMA10* |
| | H | *VMA13* |
| $V_0$ | a | *VPH1, STV1* |
| | d | *VMA6* |
| | c | *VMA3* |
| | c' | *VMA11* |
| | c'' | *VMA16* |
| | e | *VMA9* |

**Figure 8: Model of the yeast V-ATPase structure and table of the gene encoding proteins classified according to their domain belonging (adapted from (Forgac, 2007))**

# - Chapter I -

# Determination of the Role of Vacuolar Amino Acids in the EGOC-TORC1 Signalling Pathway

# I. Introduction

The yeast vacuole represents a node between amino acid metabolism and signalling. Notably, both EGOC and TORC1 localise at the vacuolar rim (Binda *et al.*, 2009; Dubouloz *et al.*, 2005; Sturgill *et al.*, 2008). Therefore, the flux of amino acids between the cytoplasm and the vacuole may be controlled by TORC1 either via its effect on autophagy or via a possible role in controlling vacuolar amino acid transporters, as suggested by phospho-proteomic studies that have identified several vacuolar amino acid transporters as being differentially phosphorylated upon rapamycin treatment (Huber *et al.*, 2009; Soulard *et al.*, 2010). Furthermore, class C-VPS mutants (*e.g. pep3*), which have impaired vacuolar functions (Bowers and Stevens, 2005), display synthetic lethality with *tor1Δ* and are rapamycin sensitive (Zurita-Martinez *et al.*, 2007). Remarkably, the growth defect of a *pep3Δ tor1Δ* mutant can be rescued by supplementation of the medium with glutamate or glutamine, establishing an additional link between nitrogen metabolism, TORC1 signalling and vacuolar functions. Of note, class C-VPS mutants have decreased levels of amino acids and especially the basic vacuolar amino acids (i.e. arginine, lysine and histidine), and are sensitive to nitrogen starvation.

Thus it appears that the vacuole plays a role as a central integrator of nitrogen availability, which prompted us to evaluate the role of the vacuole in amino acid signalling to TORC1 and vice versa. For instance, we addressed the question of which pool of amino acids, *i.e.* the vacuolar or cytoplasmic pool, plays a predominant role in activation of TORC1. We first tested whether TORC1 hyperactivation or inhibition affects the amino acid pools. Secondly, we used a set of mutants that have an altered pool of vacuolar amino acids, and tested their corresponding impact on TORC1. Finally, we investigated the link between pH and amino acid homeostasis, as vacuolar acidification is a pre-requisite for amino acid storage in the vacuole.

# II. Results

## 1. Homeostasis of the intracellular pool of amino acids

### *i Cycloheximide induces an increase of the free pool of total and vacuolar amino acids and boosts TORC1 activity*

It has been observed that cycloheximide (CHX) increases the Sch9 phosphorylation status through stimulation of TORC1 activity (Urban *et al.*, 2007) and Figure 10C). The proposed mechanism implicates a blockage of the translation process, which increases the levels of charged tRNAs to saturation, and expectedly, causes an increase of the intracellular pool of free amino acids. To confirm the latter expectation, we decided to quantify the intracellular levels of amino acids as described by Oshumi *et al.* (Kitamoto *et al.*, 1988; Ohsumi *et al.*, 1988). Basically, a $Cu^{2+}$ treatment of yeast cells induces a change in the permeability of the plasma membrane, allowing differential extraction of the vacuolar and cytosolic pools of amino acids. As it is known that the vacuole serves as an amino acid reservoir (Yang and Klionsky, 2007), we sought to analyse both cytoplasmic and vacuolar pools of amino acids to determine if one pool of amino acids would be more important for TORC1 signalling than the other one. We determined the total and vacuolar pools of amino acids, and inferred the cytoplasmic pool as the difference between the latter two pools. In order to do this, yeast cells were grown to mid-log phase, treated with cycloheximide (25 μg/mL) and samples were taken at $t_0$, $t_5$, $t_{15}$ and $t_{30}$ minutes for amino acid extraction and quantification.

Cycloheximide treatment induced a significant increase (ranging from 1.5 to 2.5 fold) of the total and vacuolar pools of amino acids already after 5 minutes and up to 30 minutes. The amino acids, whose levels were affected most dramatically (more than 1.5 fold already after 5 minutes and up to 2 fold after 30 minutes) were the two branched-chain amino acids (BCAA) leucine and isoleucine, while the levels of the two basic amino acids arginine and lysine were much less affected (less than 1.5 fold after 30 min). As basic amino acids are much more abundant in the vacuole than any other amino acid, we expected this pool to be less affected by cycloheximide treatment (Figure 9A and B).

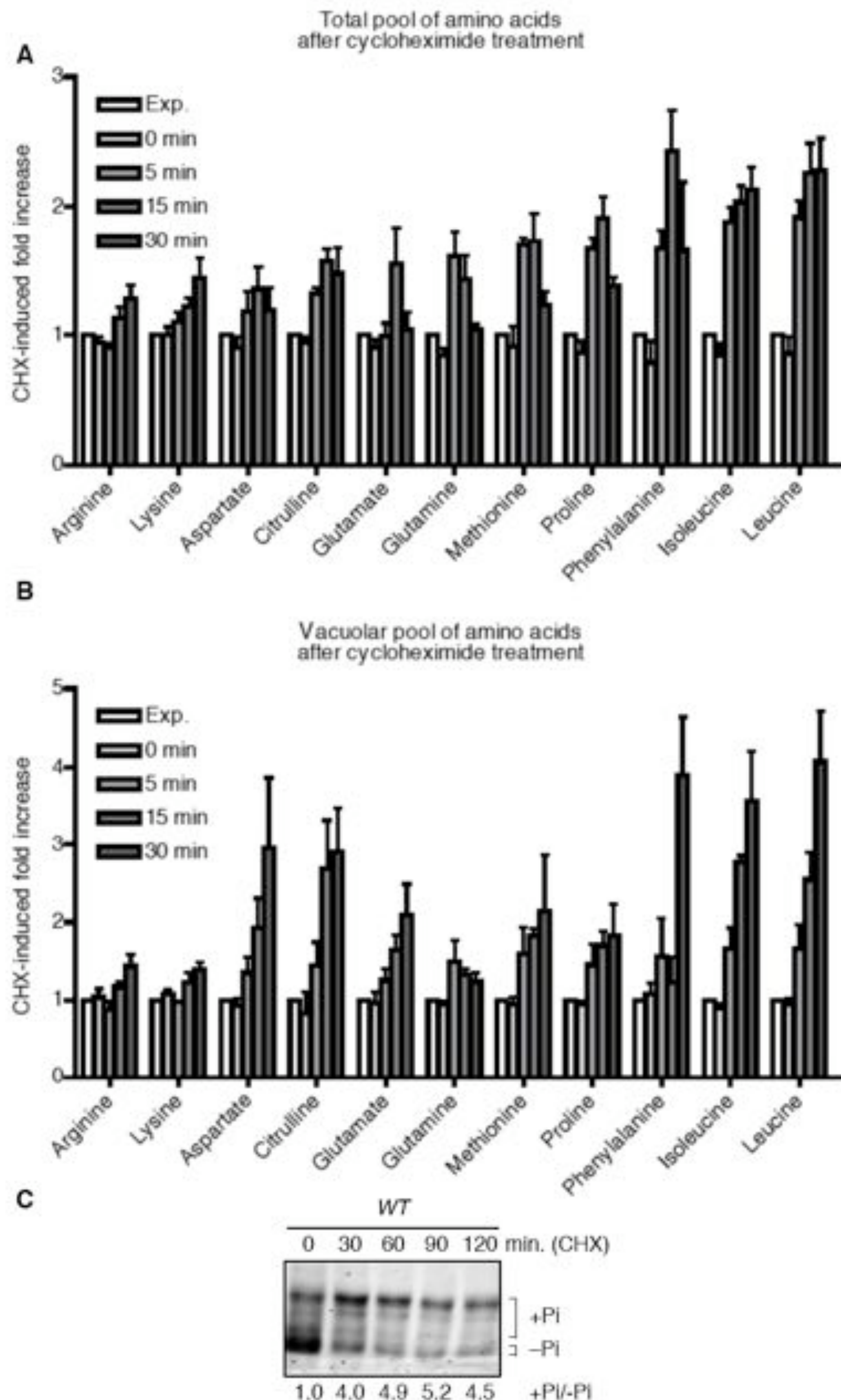



**Figure 9: Effect of cycloheximide treatment on amino acid levels and TORC1 activity over time**
(A and B): graph showing the variation of total and vacuolar amino acid pools (respectively) following cycloheximide treatment. Wild-type (WT) auxotroph cells were grown in SD medium to mid-log phase and treated with cycloheximide (25 µg/mL); samples were collected at the indicated times for amino acid extraction and quantification. The data are expressed as fold increase over the $t_0$ time point (*i.e.* before treatment) and represent the mean and standard deviation of three independent experiments**.**
(C): TORC1 activity was assessed by the level of phosphorylation of its direct downstream target Sch9. WT cells containing Sch9-T570A-$HA_5$ plasmid were grown in YPD medium to mid-log phase and treated for the indicated times with cycloheximide (25 µg/mL). Sch9 phoshorylation was measured by using NTCB chemical fragmentation analysis followed by anti-HA western immunoblotting (Urban *et al.*, 2007). Sch9 is hyperphosphorylated in response to CHX (25 µg/mL, 30 min). Relative ratios (normalized to untreated wild-type sample) between phosphorylated ($+P_i$) and dephosphorylated ($-P_i$) fractions of Sch9 are shown below the lanes.

Of note, the fact that branched chain amino acids are strongly increased in the vacuole upon cycloheximide treatment is interesting given the following observations:

- cells are highly responsive to leucine starvation (Binda *et al.*, 2009; Sancak *et al.*, 2008)
- isoleucine and leucine are scarce in the cytoplasm ((Kitamoto *et al.*, 1988), and our own experiments)
- isoleucine and leucine are abundant in proteins (Echols N. *et al.*, 2002)

Moreover, there is a clear accumulation of amino acids inside the vacuole. This indicates that the cytoplasmic pool of amino acids is tightly controlled to maintain it constant, which favours a model in which cytoplasmic amino acids may be more prone to act as a signal for TORC1. Additional experiments would be required to support this hypothesis. For example, measurement of amino acids on a longer time frame could answer the question of the homeostasis of the cytoplasmic pool. After longer times of CHX treatment, we might observe a decrease of the pool of cytoplasmic amino acids back to the level of exponentially growing cells and thus, TORC1 activity should also decrease. To test this hypothesis, we monitored the level of TORC1 activity after prolonged times of cycloheximide treatment. Up to 2 hours after addition of cycloheximide to yeast cells, Sch9 remained highly phosphorylated (4 to 5 times more than in untreated cells) (Figure 10C). From this observation, we can speculate that either the pool of cytoplasmic amino acids remains high because of the continuous uptake from the medium, or because vacuolar amino acids may also signal toward TORC1. Of note, *ego* mutants are still partially responsive to cycloheximide (Binda *et al.*, 2009) indicating that the route by which cycloheximide activates TORC1 invokes yet to be identified additional components.

Based on our experiments, we may also speculate that BCAA might be a key signal for activation of the TORC1 pathway, but this assertion would need extra experiments to be validated. It has been postulated by many that different amino acids play a prominent role in TORC1 signalling (Crespo *et al.*, 2002; Nicklin *et al.*, 2009; Sancak *et al.*, 2008) and this phenomenon seems to be conserved across evolution (De Virgilio and Loewith, 2006). The relative role of different amino acids in TORC1 signalling will be discussed in a further chapter accompanied by a series of experiments to validate or reject the hypotheses proposed in the literature (see page 64 of chapter II).

*ii TORC1 inhibition induces an increase of the total and vacuolar amino acid pools*

To further investigate the link between amino acids and TORC1, we examined the effects of direct TORC1 inhibition on the levels of amino acids. We already know that TORC1 controls translation initiation through the Gcn2 protein kinase and the Sit4-Tap42 phosphatase module (Barbet *et al.*, 1996; Cherkasova and Hinnebusch, 2003; Schmelzle and Hall, 2000). Accordingly, Gcn2 is phosphorylated on serine residue 577 in a TORC1-dependent manner, preventing its activation, while upon TORC1 inhibition, the Sit4 phosphatase gets activated and relieves the phospho-inhibition on Gcn2. Moreover, it is known that TORC1 directly controls the sorting and stability of amino acid

permeases to the plasma membrane (De Craene *et al.*, 2001; Jacinto *et al.*, 2001; Schmidt *et al.*, 1998). We may therefore speculate that, upon TORC1 inhibition, there is a change in the pattern of amino acid permeases at the plasma membrane that might modify amino acid homeostasis. To test this hypothesis, we treated yeast cells with 200 ng/mL rapamycin and quantified the levels of amino acids over time.

Rapamycin induced a cell response similar to the one of cycloheximide regarding free amino acids. Indeed, like CHX, rapamycin treatment caused a strong accumulation of total and vacuolar amino acids and particularly of the BCAA isoleucine and leucine while basic amino acids were only slightly affected (Figure 10A and B). However, as compared to cycloheximide, the kinetics of amino acid accumulation following rapamycin treatment was slower. The corresponding increase could only be observed after 15 min, but the accumulation was much higher, as it went up to 5 fold over the $t_0$ time point. In addition, the other amino acids quantified seemed to be more affected by the rapamycin than by the cycloheximide treatment.

The accumulation of amino acids following TORC1 inactivation by rapamycin may be explained by different ways, which are not exclusive. First of all, TORC1 controls amino acid uptake at the plasma membrane (De Craene *et al.*, 2001; MacGurn *et al.*, 2011; Schmidt *et al.*, 1998). Secondly, TORC1 has been shown to phosphorylate some amino acid transporters at the vacuolar rim (*e.g.* Avt1, Avt4;(Huber *et al.*, 2009; Soulard *et al.*, 2010)). This modification could modulate the activity and/or stability of these transporters. Finally, the inhibition of translation initiation following TORC1 inhibition might indirectly lead to an accumulation of free amino acids.

Thus, this set of results supports the idea that TORC1 controls the homeostasis of the amino acid pools. This assertion is complemented by the observations made in the laboratory that EGOC controls, at least partially and in a TORC1-dependent manner, the expression and/or the sorting and/or the stability of different amino acid permeases depending on the availability and the quality of the nitrogen source. Timely and accurate control of the permeases and of the amino acid vacuolar transporters likely ensures proper uptake of available amino acids and homeostasis of the intracellular cytoplasmic pool of amino acids.

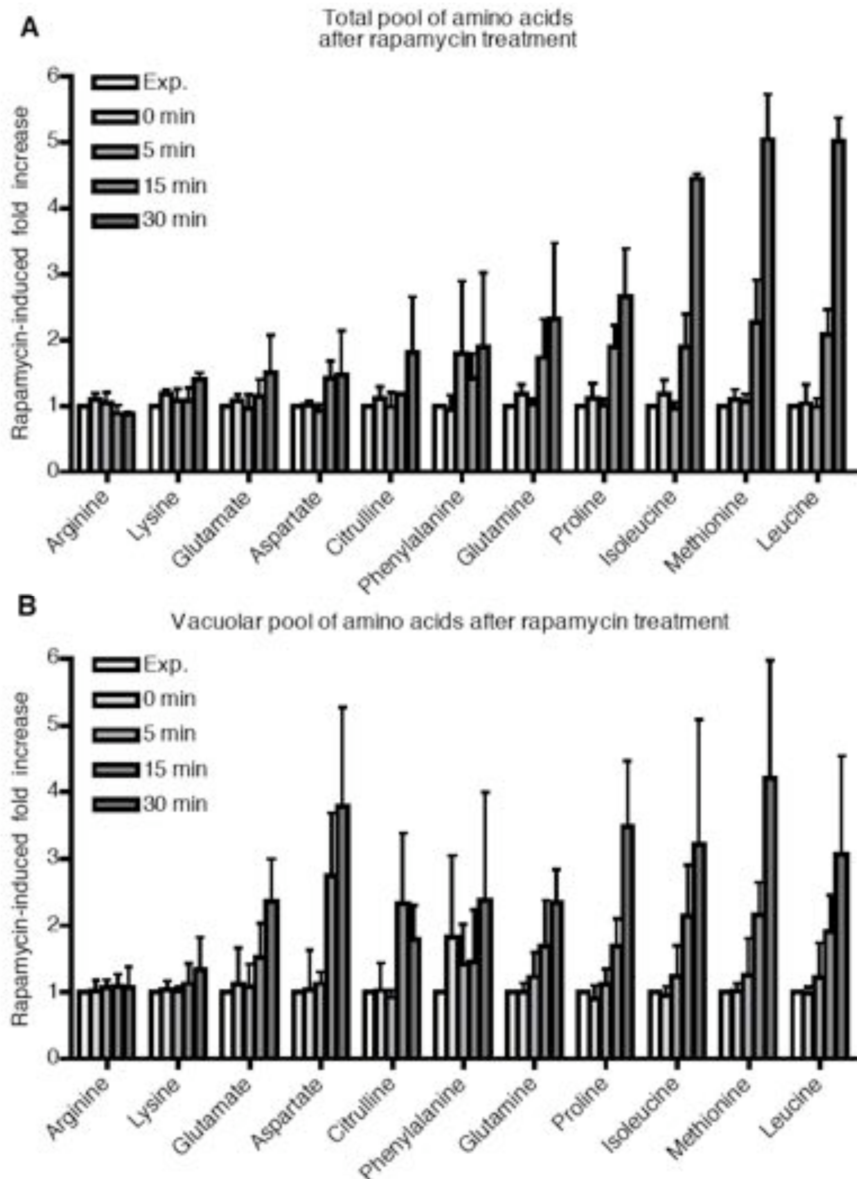



**Figure 10: Effect of rapamycin treatment on intracellular amino acid levels over time**
(A and B): graph showing the variation of total and vacuolar amino acid pools (respectively) following rapamycin treatment. Wild-type (WT) auxotroph cells were grown in SD medium to mid-log phase and treated with rapamycin (200 ng/mL); samples were collected at the indicated times for amino acid extraction and quantification. The data are expressed as fold increase over the $t_0$ time point (*i.e.* before treatment) and represent the mean and standard deviation of three independent experiments.

## 2. Effects of constitutively elevated vacuolar amino acid levels on TORC1 signalling and growth

Based on the observation that cycloheximide induces an increase in the total pool of free amino acids, and considering that most of those amino acids are taken up into the vacuole, we wanted to test the importance of the vacuolar pool of amino acids for TORC1 signalling. To this end, we used a genetic approach and deleted the genes encoding some of the main amino acids exporters at the vacuolar membrane, namely, *AVT3*, *AVT4*, *AVT6*, and *ATG22*. Avt3 and Avt4 are exporters of large neutral amino acids, especially of tyrosine, isoleucine and leucine (Russnak *et al.*, 2001; Yang *et al.*, 2006). Avt6 is responsible for the efflux of the acidic amino acids glutamate and aspartate (Russnak *et al.*, 2001), while Atg22 exports mainly leucine from the lumen of the vacuole and is functionally redundant, at least in part, with Avt3 and Avt4 (Yang *et al.*, 2006). Atg22 belongs to the family of autophagy-related proteins and its function is required for amino acid recycling from the vacuole to the cytoplasm, when autophagy is induced upon nitrogen starvation (Yang *et al.*, 2006). WT and quadruple mutant cells were grown in YPD until mid-log phase and harvested for amino acid extraction. The quadruple mutant displayed elevated levels of amino acids in the vacuole as compared to the WT but more particularly of the amino acids leucine and isoleucine (Figure 11A and B). This result is in line with what was already described in a *avt3Δ avt4Δ atg22Δ* mutant (Yang *et al.*, 2006). However, in the latter study, the triple mutant was mainly affected for tyrosine, which is one of the amino acids hardly separated with our HPLC system and, as a consequence, not present in our quantifications. Additionally, we observed an increase in the vacuolar levels of glutamate and aspartate which might be accounted for, at least in part, by the effect of *avt6* deletion (Russnak *et al.*, 2001). The increased levels of citrulline were never described before.

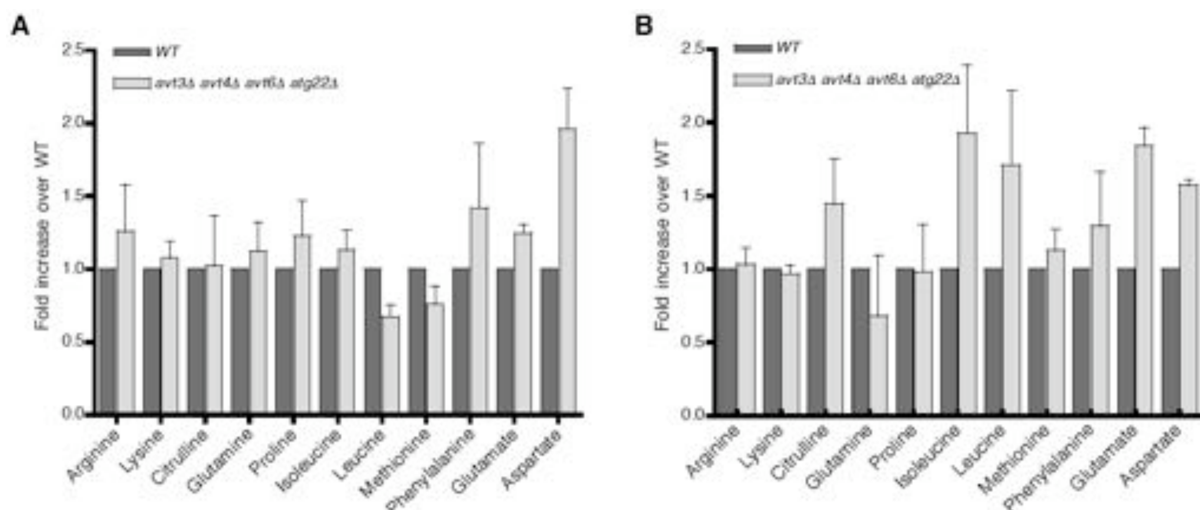



**Figure 11: *avt3Δ avt4Δ avt6Δ atg22Δ* mutant displays elevated levels of amino acids inside the vacuole**

(A and B): graph showing the variation of the total (A) and vacuolar (B) amino acid pools of WT and *avt3Δ avt4Δ avt6Δ atg22Δ* yeast strains. WT and *avt3Δ avt4Δ avt6Δ atg22Δ* auxotroph cells were grown in SD medium to mid-log phase and samples were collected at the indicated times for amino acid extraction and quantification. The data are expressed as fold increase over the $t_0$ time point (*i.e.* before treatment) and represent the mean and standard deviation of three independent experiments.

We then tested the resistance of single and multiple deletion strains to low levels of rapamycin. We know that deletion of any of the genes encoding EGOC components confers hypersensitivity to rapamycin and over-expression of the GTP or GDP restricted alleles of Gtr1 or Gtr2, respectively, confers partial resistance to rapamycin (Binda *et al.*, 2009). This phenotypic analysis gives us information on a possible role of vacuolar amino acids in TORC1 signalling. Deletion of each single exporter did not affect rapamycin sensitivity on plate as compared to the isogenic WT strain, but deletion of *avt3* together with *avt4* or *avt3*, *avt4* and *avt6* and deletion of the four exporters conferred a significant resistance to rapamycin at doses up to 100 ng/mL (Figure 12A). Usually, this concentration of rapamycin is toxic to WT cells and completely inhibits growth. This is a first indication that vacuolar amino acids may sustain growth by activating TORC1. To better understand how the levels of vacuolar amino acids might affect growth on rapamycin plates, we monitored the level of Sch9 phosphorylation in exponentially-growing cells and in rapamycin- or cycloheximide-treated cells. We hypothesized that maintaining high levels of amino acids in the vacuole might directly or indirectly increase TORC1 activity and thus, mimic the effect of a cycloheximide treatment on TORC1 activity in exponentially growing cells. However, we could not observe any significant differences between wild-type and any of the tested mutants with respect to TORC1 activity (Figure 12B).

Therefore, we decided to assess the activity of TORC1 in the quadruple mutant strain under two conditions:

- with increasing concentrations of rapamycin
- upon leucine starvation

Under both conditions, the quadruple *avt3*, *avt4*, *avt6*, and *atg22* mutant strain behaved as a WT strain (Figure 13), indicating that the partially rapamycin resistant growth phenotype of the quadruple mutant may not result from a protective effect of vacuolar amino acid under stressful conditions on TORC1 itself. Even though the cells are in distinct physiological states in the two assays, the fact that accumulation of amino acids inside the vacuole protects the cells, to some extent, from a rapamycin-induced growth arrest obviously indicates that this pool of amino acids plays a role in the control of cell growth.

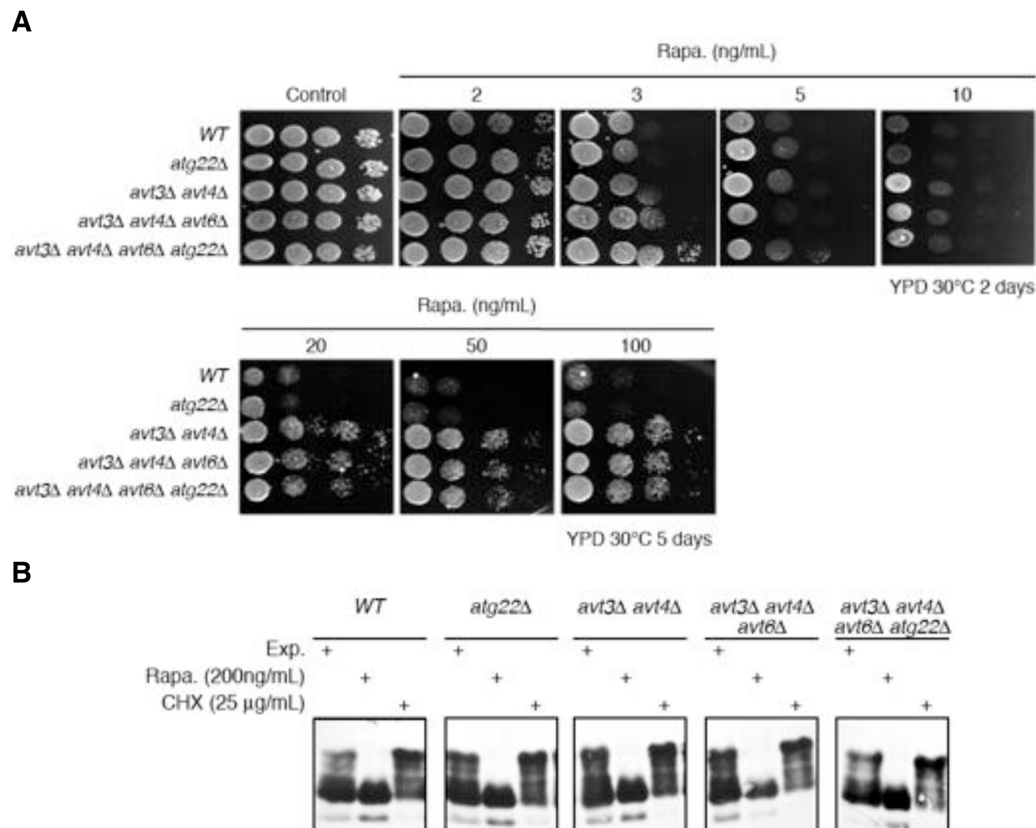



**Figure 12: Loss of Avt3, Avt4, Avt6 and Atg22 improves growth on rapamycin plates but do not directly impinge on TORC1 activity**

(A): *avt3Δ avt4Δ avt6Δ atg22Δ* mutant shows rapamycin resistance on plates. Serial 10-fold dilutions of WT and mutants auxotroph cells were spotted on YPD plates containing the indicated concentrations of rapamycin, and allowed to grow either for 2 or 5 days as indicated.

(B): TORC1 activity was assessed by the level of phosphorylation of its direct downstream target Sch9 in exponential phase or upon rapamycin or cycloheximide treatment. Cells were grown in YPD medium and treated for 30 min with the indicated concentration of drug.

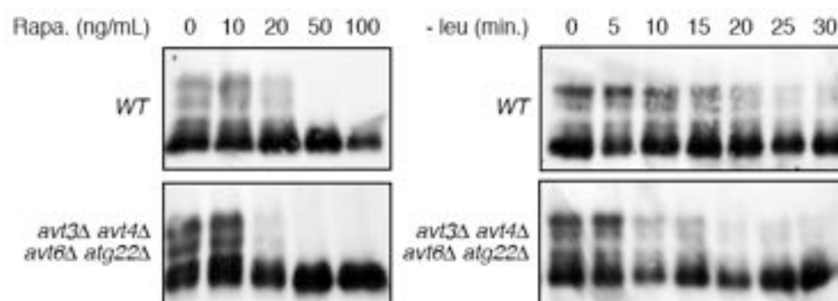



**Figure 13: Loss of Avt3, Avt4, Avt6 and Atg22 does not prevent TORC1 inhibition following rapamycin treatment or leucine starvation**

Sch9 phosphorylation status was assessed in leucine auxotrophic WT and mutant cells grown in SD medium supplemented with leucine and treated with increasing concentrations of rapamycin or following a 30 min time-course of leucine deprivation.

In order to address a possible role for vacuolar amino acids in TORC1 activation via EGOC, we further deleted *gtr1* in the quadruple mutant background and performed the same experiments (i.e. growth assay on rapamycin plates and assessment of TORC1 activity). The quintuple mutant was similarly sensitive to rapamycin, as a *gtr1Δ* single mutant (Figure 14A), indicating that *gtr1* is epistatic to the vacuolar amino acid exporters. Furthermore, TORC1 activity in the quintuple mutant was similar to that of a single *gtr1Δ* mutant (Figure 14B). All together, our data rather indicate that the vacuolar exporters studied may potentially work downstream of or in parallel to TORC1.

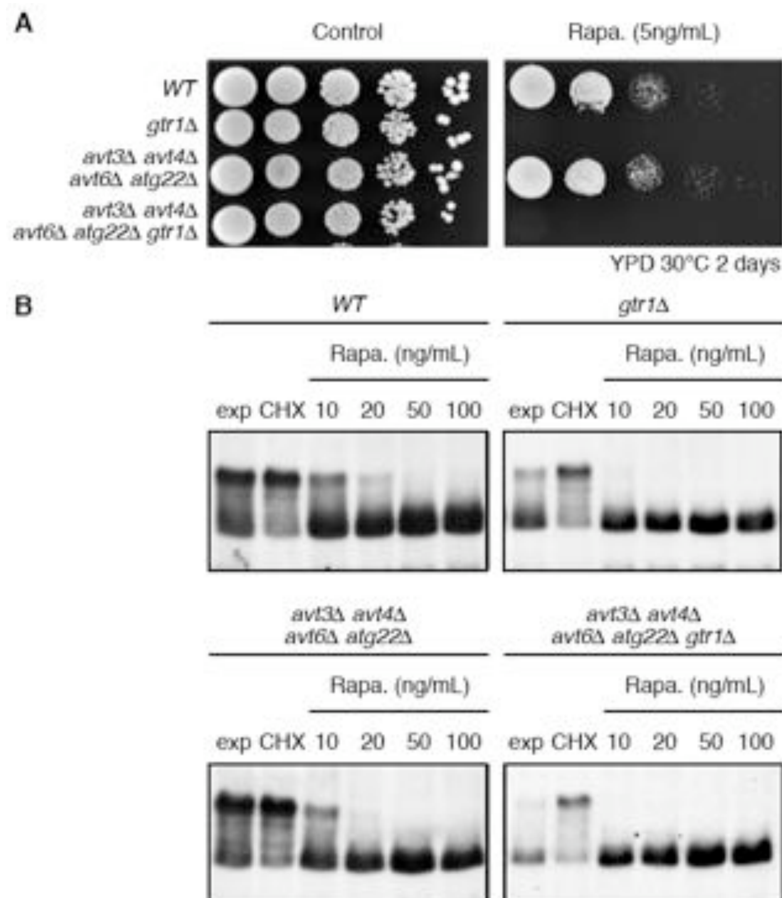



**Figure 14: *gtr1 is* epistatic to the vacuolar amino acid exporters**
(A) Deletion of gtr1 in the quadruple mutant background mimics gtr1Δ phenotype on rapamycin-containing plate. Serial 10-fold dilutions of WT and mutants cells were spotted on plates containing the indicated concentrations of rapamycin and grown for two days.
(B): Deletion of gtr1 in the quadruple mutant background mimics gtr1Δ phenotype regarding TORC1 activity. Sch9 phosphorylation status was assessed as described earlier (see Figure 10C). Yeast cells with the indicated genotype were grown in SD medium to mid-log phase (exp) and treated for 30 min with cycloheximide (25 mg/mL) or with the indicated concentrations of rapamycin.

Finally, Atg22 has been found to be essential for viability upon starvation. Deletion of *atg22*, together with *avt3* and *avt4*, decreases the chronological lifespan (CLS) of yeast cells following a prolonged starvation and this effect can be bypassed by addition of leucine to the medium (Yang *et al.*, 2006). To broaden the result obtained by Yang and co-workers, we evaluated the chronological lifespan of the *avt3Δ avt4Δ avt6Δ atg22Δ* mutant. Even though there is no obvious up-regulation of

TORC1 activity in the quadruple mutant, we assumed that it might have a decreased lifespan. It has been clearly established that hyperactivation of TORC1 increases rapamycin sensitivity of yeast cells and decreases CLS, while down-regulation of TORC1 prolongs yeast CLS (Fabrizio *et al.*, 2001; Wanke *et al.*, 2008). We hypothesised that the rapamycin resistance phenotype in our quadruple mutant might correlate with a decreased longevity, which could have indicated that longevity is regulated by a mechanism that is, at least, partially independent of TORC1. As a consequence, WT and *avt3Δ avt4Δ avt6Δ atg22Δ* cells were grown in YPD, and 10-fold serial dilutions were plated every 3 days on YPD. Colony forming units (c.f.u.) were counted and the result expressed as % of survival. We did not observe differences in the survival rate of the *avt3Δ avt4Δ avt6Δ atg22Δ* mutant as compared to the WT. Nonetheless this result is in line with our observation that TORC1 activity is not affected in the quadruple mutant (Figure 15).

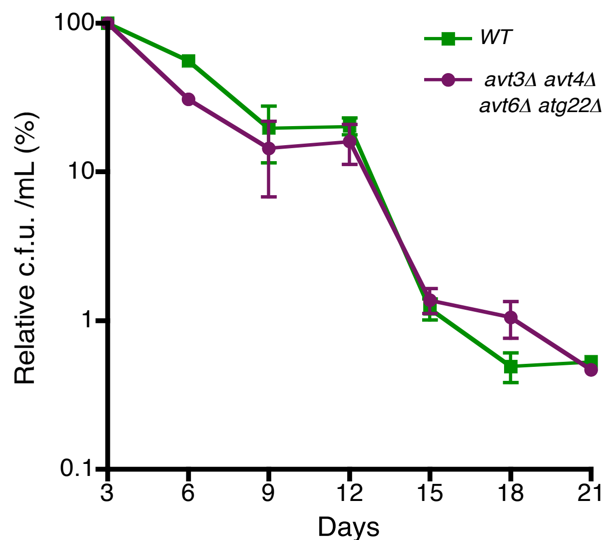



**Figure 15: Vacuolar amino acids do not affect the chronological lifespan of yeast.**
The quadruple vacuolar amino acid exporter mutant displays the same chronological lifespan as WT cells. Each data point represents the mean of three samples. Survival data (cfu $ml^{-1}$) are expressed as relative values compared with the values at day 3 (early stationary phase).

In our experiments, we identified the vacuolar pool of amino acids as a modulator of cell growth. Nevertheless, vacuolar amino acids do not appear to play a role in TORC1 activity control. We can formulate hypotheses concerning the route by which this particular pool of amino acids sustains growth under stressful conditions. Vacuolar amino acids might control TORC1-downstream effectors such as the GATA transcription factors Gat1 and Gln3, which control, the expression of several target genes encoding permeases and metabolic enzymes (Courchesne and Magasanik, 1988; Stanbrough *et al.*, 1995). Of note, the quadruple *avt3Δ avt4Δ avt6Δ atg22Δ* mutant accumulates glutamate in the vacuole (Figure 11), and could consequently have a constitutive activation of the RTG pathway, which might give this mutant an advantage under certain conditions. TORC1 is a known regulator of the NCR and RTG transcription factors in response to the quality and quantity of the amino acid source (Beck and Hall, 1999; Dilova *et al.*, 2004), but it is still possible that the

function of these factors is controlled independently of TORC1. It was proposed that TORC1 control of *RTG* gene expression may derive indirectly from alterations in nitrogen metabolism rather than direct TORC1 regulation (Tate and Cooper, 2003). Thus, localisation studies of the corresponding transcription factors in WT and *avt3Δ avt4Δ avt6Δ atg22Δ* may give clues for a role of vacuolar amino acids in the control of cell growth. Of note, *gln3Δ* cells are resistant to rapamycin (Cardenas *et al.*, 1999) but do not display a defect in TORC1 activity. Moreover, a *gln3Δ gtr1Δ* double mutant is able to recover from a rapamycin-induced growth arrest, although its TORC1 activity is similar to the one of a *gtr1Δ* single mutant (Binda *et al.*, 2009). Thus, epistasis analysis should give valuable information regarding the role of vacuolar amino acids in NCR gene expression.

Independently of amino acid-regulated transcription factors, the targeting and stability of permeases at the plasma membrane might be affected in an *avt3Δ avt4Δ avt6Δ atg22Δ* mutant. For instance, the nitrogen permease regulator Npr1 controls the stability of amino acid permeases at the plasma membrane in a TORC1-dependent manner and *npr1Δ* cells also display a rapamycin resistance phenotype on plates, but should not present an altered TORC1 activity, as Npr1 acts downstream of TORC1 (De Craene *et al.*, 2001; MacGurn *et al.*, 2011; O'Donnell *et al.*, 2010; Schmidt *et al.*, 1998).

### 3. The yeast V-ATPase is not a sensor of amino acids

During the course of our search for possible sensors of intracellular amino acids, Zoncu and colleagues found that the mammalian V-ATPase is necessary to activate mTORC1 in response to lysosomal amino acids, through amino acid-sensitive interactions with the Rag GTPases/Ragulator complex (Zoncu *et al.*, 2011).

In order to test this possibility in yeast, we decided to use two different approaches:

- a chemical approach using concanamycinA, a well described inhibitor of the $V_0$ subunit of the V-ATPase (Drose *et al.*, 1993; Nishihara *et al.*, 1995)
- a genetic approach making use of mutants of the V-ATPase subunits ($V_0$ or $V_1$)

First of all, TORC1 activity was not affected in exponentially growing yeast cells treated with concanamycin A at doses ranging from 10 nM up to 1 mM (Figure 16A). At this concentration of concanamycin A, the V-ATPase is completely inhibited and the vacuolar lumen is not acidified anymore (Baars *et al.*, 2007). To test the efficiency of the drug in our working conditions, we stained yeast cells treated with 1 mM Concanamycin A with quinacrine. Quinacrine is a known fluorogenic compound commonly used to stain acidic compartments of a cell and is consequently the compound of choice to stain yeast vacuoles (Krasowska *et al.*, 2004). As we can see in Figure 16B, WT cells

accumulated quinacrine inside the vacuole while both V-ATPase mutant and WT cells treated with ConA failed to do so, indicating that their vacuoles were not properly acidified, as expected.

To further confirm that V-ATPase impairment does not affect TORC1 signalling, we decided to assess the Sch9 phosphorylation status in mutants of the V-ATPase. We chose to test different mutants both in the $V_0$ (*vma3Δ* and *vma6Δ*) and $V_1$ (*vma1Δ*, *vma2Δ* and *vma8Δ*) subunits. V-ATPase mutants displayed a TORC1 activity similar to that of the WT (Figure 17C). This result differs from the one of Zoncu and colleagues who showed that, in *Drosophila* S2 cells, knock-down of the vhaAC39 and vha16 subunits (respective homologues of yeast Vma6 and Vma3) of the V-ATPase, decreases dTORC1 activity as efficiently as knock-down of dRagC under nutrient rich conditions (Zoncu *et al.*, 2011).

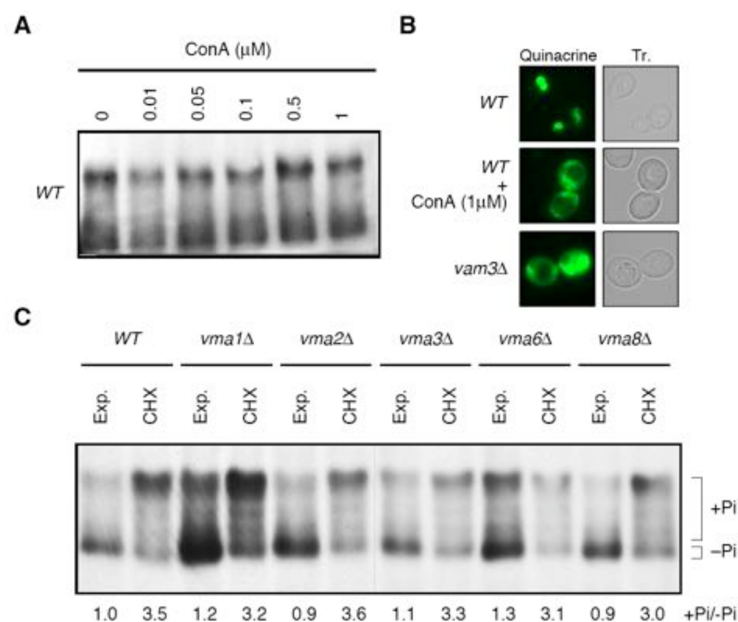



**Figure 16: Impairment of V-ATPase function does not affect TORC1 activity**
(A and B) Treatment with concanamycin A does not impact on TORC1 activity although it affects V-ATPase.
(A) Cells were grown to mid-log phase in SD medium and treated with increasing concentrations of concanamycin A. Quinacrine staining was performed on indicated strains treated with concanamycin A for 30 min as described by Krasowska (Krasowska *et al.*, 2004).
(C) V-ATPase mutants display a TORC1 activity comparable to their isogenic WT and are still responsive to CHX treatment. Yeast cells were grown to mid-log phase in SD medium and assessed for TORC1 activity prior to and following a 30 min CHX treatment (25mg/μL). Relative ratios (normalized to untreated wild-type sample) between phosphorylated and dephosphorylated fractions of Sch9 are shown below the lanes, as described previously.

Moreover, yeast V-ATPase mutants were still responsive to cycloheximide (Figure 16C), indicating that, if intracellular amino acids were to be sensed through the V-ATPase in yeast, it is not a unique mechanism. It is also possible that this mechanism appeared later during evolution and is specific to higher eukaryotes. On the other hand, a recent genetic screen in *Drosophila* identified two proton-dependent high affinity amino acid transporters, PAT1 and PAT4, that localise to endosomal membrane and that are important modulators of TORC1-mediated growth (Heublein *et al.*, 2010). Notably the study showed that:

- in *Drosophila*, expression of PAT1 and PAT4 promotes growth through dTORC1.
- PAT1 and PAT4 control growth and proliferation in MCF-7 breast cancer cell line through mTORC1
- PAT1 and PAT4 modulate mTORC1 response to amino acids in HEK-293 cells

This study raises the possibility that Zoncu and colleagues observed rather indirect effects due to the loss of acidification of the lysosomes, and impairment of the global pH homeostasis of the cell. Notably, V-ATPases localize both on lysosomes and at the plasma membrane in HEK-293T cells (Alzamora *et al.*, 2010). In mammalian cells, pH homeostasis is required for a variety of cellular processes, including receptor-mediated endocytosis, protein degradation in lysosomes, processing of hormones, uptake and storage of neurotransmitters and various metabolic reactions (for review see (Stevens and Forgac, 1997)).

The drug niclosamide was previously shown to inhibit mTORC1 signalling (Balgi *et al.*, 2009). While investigating the mechanism by which niclosamide affects mTORC1, Fonseca *et al.* nicely demonstrated, in a very recent study (Fonseca *et al.*, 2012), that cytoplasmic acidification inhibits mTORC1 signalling. Niclosamide is a salicylanilide originally used to cure tapeworm infections and was proposed to do so by disrupting the pH homeostasis of the parasite (Fairweather and Boray, 1999). Fonseca and colleagues established that niclosamide is a protonophore, whose capacity to embed in membranes and to rapidly and reversibly bind protons, disrupts the proton gradient across membranes. The consequences of such proton gradient impairment is an acidification of the cytosolic pH by up to 0.5-1 pH units and a subsequent inhibition of mTORC1 signalling, without affecting the synthesis of ATP as judged by the levels of cellular ATP (Balgi *et al.*, 2009; Fonseca *et al.*, 2012). In addition, disruption of the cytoplasmic pH by alternative means led to the same results, i.e. inhibition of mTORC1, while impairment of the lysosomal pH had no effect on mTORC1 activity (Fonseca *et al.*, 2012).

It has been shown that glucose deprivation induces a reversible disassembly of the $V_0$ and $V_1$ subunit of the V-ATPase. Thus, we might imagine a similar mechanism following amino acid deprivation. This mechanism would block the proton-coupled antiport of amino acids into the vacuole. However, addition of cycloheximide, which stimulates TORC1 activity, has no impact on the assembly nor disassembly of the V-ATPase (Kane, 1995). So, it seems unlikely that TORC1 regulates V-ATPases in response to amino acids.

Finally, mTORC1 controls the phosphorylation and nuclear localisation of the transcription factor EB (TFEB), which controls in turn the expression of a set of lysosomal genes, notably, V-ATPase genes (Pena-Llopis *et al.*, 2011; Settembre *et al.*, 2012). Thus, it seems that lysosomal acidification and mTORC1 activity are closely intertwined.

# III. Discussion

In this chapter, we tried to define the role of the vacuolar amino acids in TORC1 signalling. Through different approaches, we have shown that inhibition of TORC1 with rapamycin, as well as hyperactivation of TORC1 with cycloheximide, induce dramatic changes in the different pools of intracellular amino acids, especially of the branched-chain amino acids. We have also seen that the vacuolar pool of amino acids plays a role in the control of cell growth, although it does not appear to act on the EGOC-TORC1 signalling pathway. Finally, by using both chemical and genetic approaches, we demonstrated that in yeast, unlike what has been proposed in mammals (Zoncu *et al.*, 2011), the V-ATPase is not required for vacuolar amino acid sensing by TORC1.

Although there is no evidence for an influence of TORC1 on vacuolar amino acid permeases, some of these transporters were found to be phosphorylated in a TORC1-dependent manner (Huber *et al.*, 2009). Moreover, TORC1 controls the permease composition at the plasma membrane by negatively regulating Npr1 (MacGurn *et al.*, 2011). This provides a model in which amino acid control of TORC1 modulates the fluxes of amino acids in the cell and, hence, growth according to amino acid availability.

Currently, our data presented in this chapter do not allow us to draw any conclusions regarding the importance of each pool of intracellular amino acids in the control of cell growth and/or TORC1. Storage of vacuolar amino acids is particularly important for cells to survive following long-term starvation, and the vacuolar pool may play a role by providing the missing amino acids, and more specifically leucine, required to maintain a minimal metabolism (Yang *et al.*, 2006). The exact role of vacuolar amino acids in cell growth control are unclear, but the observation that they sustain growth following partial TORC1 inactivation opens the question of the existence of an alternative signalling pathway that may converge on some already known TORC1 downstream effectors, most probably transcription factors that couple cell growth to the nitrogen source.

The role of leucine is of particular interest given its accumulation in the vacuole following treatment with drugs that affect oppositely TORC1 activity (rapamycin and cycloheximide) or following loss of the corresponding amino acid exporters. Previous studies already reported that leucine is an intermediate quality nitrogen source (Magasanik, 1992), is required for long-term survival following amino acid starvation (Yang *et al.*, 2006) and is a potent activator of TORC1 (Binda *et al.*, 2009). Nevertheless, leucine is not the preferred nitrogen source and, as such, glutamine would also play an important role in cell growth control in response to amino acids (Binda *et al.*, 2009; Crespo *et al.*, 2002; Magasanik and Kaiser, 2002).

The means by which amino acids activate EGOC to stimulate TORC1 activity and sustain growth remains unclear. Moreover, the direct role of amino acids in stimulating Gtr1 has to be proven and it is possible that they act indirectly through other ways. The amino acid biosynthetic pathways are inter-connected with many other pathways, from nucleotide biosynthesis to tricarboxilyc cycle

and mitochondrial respiration. These possible aspects of amino acid sensing and regulation of the TOR pathway will be discussed in the next chapter.

# - Chapter II -

# Determination of the Role of the TCA Cycle and of Glutamine in the EGOC-TORC1 Signalling Pathway

# I. Introduction

The tricarboxylic acid cycle is a key node that integrates both nitrogen and carbon sources to function. Per glucose molecule, it produces 4 $CO_2$, 2 GTP, 6 NADH, and 2 dihydroquinone. The NADH provided by the TCA cycle serves in part for oxidative phosphorylation, a metabolic pathway that uses the energy released by oxidation of nutrients to produce ATP. During one cycle, $\alpha$-ketoglutarate is generated, which can be converted to glutamate or glutamine following incorporation of one or two $NH_3$, respectively. Additionally, the succinate produced by the TCA cycle is further used by the complex II of the respiratory chain to generate the proton gradient that is required for the mitochondrial F-ATPase to function. Thus, this cycle is central to the metabolism and is able to respond to both nitrogen and carbon limitations, hence could act as an amino acid/carbon sensor. This assumption is also based on the fact that different screens performed previously in the laboratory identified genetic interactions between EGOC components and genes involved in mitochondrial functions. For example, loss of *RTG2* or *RTG3* is synthetic lethal with loss of *GTR2* and this lethality can be rescued by addition of glutamate to the medium. On the other hand, *GDH3* and *GLN1*, acting in glutamine biosynthesis, were identified as dosage suppressors of the rapamycin sensitivity of *gtr2Δ*. Furthermore, *PRS4* and *ADE2*, implied in GTP biosynthesis, were also identified as dosage suppressors of the rapamycin sensitivity of *gtr2Δ* (Dubouloz *et al.*, 2005); Binda, personal communication), and glutamine is required for the synthesis of purine nucleotides. Moreover, the histidine and nucleotide biosynthetic pathways are connected. It is thus tempting to make a link between TORC1 and purine biosynthesis and test whether inhibition of the latter could impact on the former.

Thus, to test whether the EGO complex could integrate direct or indirect signals from the TCA cycle, we decided to test the three following drugs:

- Methionine sulfoximine is a well-characterized inhibitor of the glutamine synthetase Gln1. It has already been proposed that intracellular glutamine might act as a key signal for TORC1 activation (Crespo *et al.*, 2002). Moreover, leucine, the most potent activator of TORC1 is involved in glutamine biosynthesis, and glutamine partially overcomes the effects of leucine starvation (Binda *et al.*, 2009).
- Antimycin A is a known inhibitor of the mitochondrial respiratory chain. A blockage in this process might lead to a consequent blockage of the TCA cycle through accumulation of succinate and oxidized $FAD^+$ and a concomitant decrease in glutamate and glutamine levels.
- Mycophenolic acid (MPA) inhibits the inosine monophosphate dehydrogenase that catalyses the limiting step in GTP biosynthesis that requires glutamine to function.

# II. Results

## 1. Evaluation of the role of glutamine in TORC1 signalling

It has been suggested that glutamine is a key metabolite controlling the TORC1 pathway (Butow and Avadhani, 2004; Crespo *et al.*, 2002; Dubouloz *et al.*, 2005; Urban *et al.*, 2007). This hypothesis was formulated based on the following observations:

- methionine sulfoximine (MSX), an inhibitor of the glutamine synthetase Gln1 that mimics glutamine starvation, induces nuclear localisation of the transcription factor Gln3 (but not Gat1), like rapamycin, and this event depends on the Sit4 phosphatase (Crespo *et al.*, 2002).
- *gln3* deletion confers rapamycin resistance, but MSX sensitivity on plates (Crespo *et al.*, 2002; Cardenas *et al.*, 1999).
- Gln3 is phosphorylated in a TORC1-dependent manner and dephosphorylated upon MSX treatment, as it is upon rapamycin treatment (Bertram *et al.*, 2000; Crespo *et al.*, 2002).
- yeast cells grown with glutamine as the sole source of nitrogen respond strongly to glutamine deprivation with respect to TORC1 activity (Urban *et al.*, 2007).

To assess the importance of glutamine regarding TORC1 signalling pathway, we spotted exponentially growing WT cells or *gtr1Δ* cells expressing the different alleles of Gtr1 (Gtr1, $Gtr1^{GTP}$, $Gtr1^{GDP}$) from the $Tet_{ON}$ promoter on SD medium containing increasing amounts of MSX. As described previously by Crespo and colleagues (Crespo *et al.*, 2002), MSX affects the growth of WT cells on plates already at a concentration of 200 μM, but this effect was not bypassed by overexpression of $Gtr1^{GTP}$ (Figure 17A). However, the effect of MSX conferred lethality to *gtr1Δ* cells expressing the GDP-restricted form of Gtr1, which might be an indication that glutamine signals in parallel to EGOC.

To determine whether glutamine depletion might directly affect TORC1 activity, we assessed the level of phosphorylation of Sch9 upon MSX treatment (Urban *et al.*, 2007). WT yeast cells were grown in selective medium to mid-log phase prior to addition of increasing amounts of MSX for 30 minutes. As expected, MSX blocked the phosphorylation of Sch9 (Figure 17B) in a concentration-dependent manner, and the effect was as strong as the effect of glutamine deprivation (Urban *et al.*, 2007) or leucine deprivation (Binda *et al.*, 2009). This confirms that glutamine is a very potent activator of TORC1 signalling. EGOC plays a crucial role in relaying the amino-acid signal to TORC1, and Gtr1, in its GTP-bound state, directly activates TORC1. Moreover, upon leucine deprivation, over-expression of $Gtr1^{GTP}$ partially prevents inactivation of TORC1 (Binda *et al.*, 2009). Thus, we over-expressed $Gtr1^{GTP}$ in cells grown to mid-log phase in selective medium prior to MSX treatment, and took samples every 15 min. The kinetic of inhibition of TORC1 remained the same as in WT cells (Figure 17C), indicating that the MSX effect on TORC1 activity is independent of the EGOC.

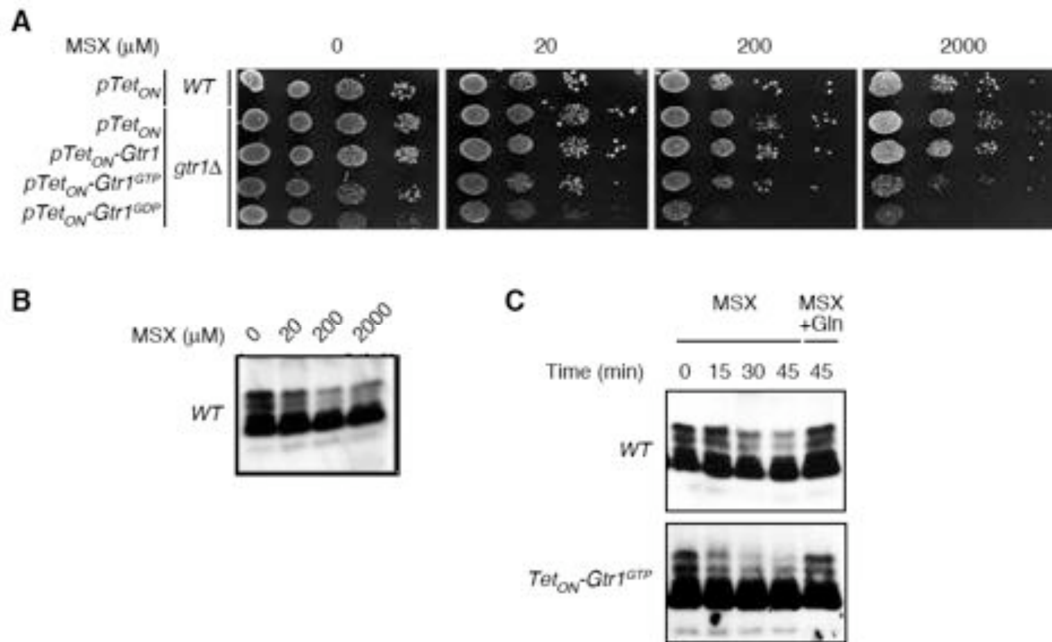



**Figure 17: Methionine sulfoximine (MSX) inhibits growth and TORC1 activity independently of the EGOC**

(A) MSX slightly inhibits growth of WT and *gtr1Δ* cells expressing the different alleles of Gtr1. Serial 10-fold dilutions of WT and mutants cells were spotted on SD plates containing the indicated concentrations of the drug and doxycyline at a final concentration of 5 µg/mL, and grown for 2 days.
(B) WT cells were grown in SD medium and treated for 30 min with the indicated concentrations of the drug.
(C) WT cells expressing vector (WT) or Gtr1$^{GTP}$ from the doxycycline inducible promoter (Tet$_{ON}$-Gtr1$^{GTP}$) were grown in SD medium in the presence of doxycycline (5 µg/mL) to mid-log phase. Upon addition of MSX (2 mM), time-points were taken and proteins extracted for Sch9 phosphorylation analysis.

As previously observed (Crespo *et al.*, 2002), addition of glutamine to cells treated with MSX reactivates TORC1, indicating that the drug effectively depletes the intracellular pool of glutamine. Although glutamine availability controls Gln3 localisation and activity in a TORC1-dependent manner, it also acts on the transcription factors Rtg1 and Rtg3. However, this regulation of the retrograde (RTG) pathway seems to be only partially dependent on TORC1, as glutamate can prevent activation of the pathway upon rapamycin treatment (Dilova *et al.*, 2004; Tate and Cooper, 2003). Notably, rapamycin treatment and glutamine starvation differentially affect the Rtg3 phosphorylation pattern (Dilova *et al.*, 2004; Komeili A. *et al.*, 2000). Moreover, loss of mitochondrial functions activates the RTG pathway but not the NCR pathway, indicating that RTG and TORC1 pathways act in parallel to converge on Rtg1/3 (Giannattasio *et al.*, 2005).

The TORC1 subunit Lst8 seems to play an important role in response to glutamine depletion. Analysis of *lst8* alleles led to the observation that this protein acts both upstream and downstream of Rtg2 to control Rtg1/3-dependent genes (Chen and Kaiser, 2003; Liu *et al.*, 2001). Apart from glutamine depletion, histidine starvation can also activate the RTG pathway independently of the GAAC (Giannattasio *et al.*, 2005). It seems that glutamate and glutamine are important for mitochondrial functions and may serve as a signal to couple the amino acid availability and the

energy status of the cell. The mechanisms by which TORC1 senses amino acids, and particularly glutamine, are still poorly understood.

## 2. Evaluation of the role of the respiratory chain in TORC1 signalling

Succinate is a product of the TCA cycle, which transmits electrons to the electron transport chain, and thus participates in the oxidative phosphorylation. As for glutamate, we hypothesized that a block in the respiratory chain would induce an accumulation of succinate, a concomitant stop in the TCA cycle, and finally a decrease of the synthesis of glutamate and glutamine and in the production of energy.

To assess the importance of mitochondrial functions for TORC1 signalling, we decided to test growth on Antimycin A, an inhibitor of the cytochrome c reductase in the mitochondrial respiratory chain. WT cells or *gtr1Δ* cells expressing the different alleles of Gtr1 (Gtr1, $Gtr1^{GTP}$, $Gtr1^{GDP}$) from the $Tet_{ON}$ promoter were spotted on YP Glycerol/Ethanol medium containing increasing amounts of Antimycin A. Antimycin A had a very slight growth inhibitory effect on plates and its effect was not bypassed by over-expression of Gtr1 or $Gtr1^{GTP}$ (Figure 18A). Additionally, at doses that were shown to affect the mitochondrial ATPase (Lefebvre-Legendre *et al.*, 2003), we could not observe any inhibition of TORC1 activity (Figure 18B).

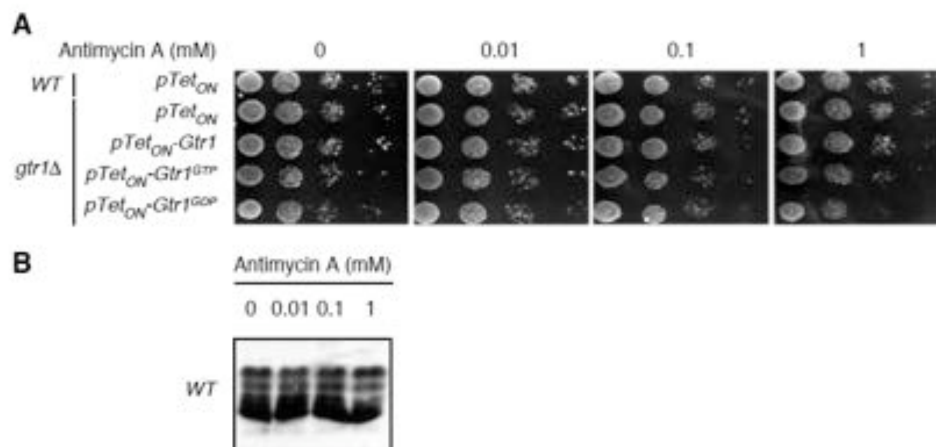



**Figure 18: Effects of Antimycin A treatment on growth and TORC1 activity**
(A): Antimycin A slightly inhibits growth of WT and *gtr1Δ* cells expressing the different alleles of Gtr1. Serial 10-fold dilutions of WT and mutants cells were spotted on YP Glycerol/Ethanol plates containing the indicated concentrations of antimycin A, and doxycycline at a final concentration of 5 µg/mL, and grown for two days.
(B): TORC1 activity was assessed by the level of phosphorylation of its direct downstream target Sch9 upon Antimycin A treatment. Cells were grown in YP Glycerol/Ethanol medium and treated for 30 minutes with the indicated concentrations of the drug.

In a recent publication, Kawai and colleagues have shown that respiratory deficient ($\rho^0$) cells display low TORC1 activity (Kawai *et al.*, 2011). This result seemingly contrasts with our observation that Antimycin A does not affect TORC1. However, the effects of Antimycin A on growth were very weak and we cannot rule out that the condition in which the drug was tested were sub-optimal (*e.g.* pH of the medium). Thus, additional experiments are required to establish that

Antimycin A inhibited mitochondrial functions in our conditions. Notably, activation of the RTG pathway could be easily tested, for example by assessing the localization of the transcriptional activator Rtg1-Rtg3 or by monitoring the expression level of RTG specific genes. However, in their study, Kawai *et al.* showed that the deficiency of $\rho^0$ mutants in activating TORC1 is independent of the EGOC, as over-expression of $Gtr1^{GTP}$ does not restore TORC1 activity. This point is also discussed by Kawai and colleagues who additionally noted that, in the presence of a $Gtr1^{GTP}$ allele, Sch9 phosphorylation is lower in *gtr1Δ* $\rho^0$ than in *gtr1Δ* cells. They also concluded that mitochondrial dysfunction is probably not conveyed to TORC1 through an amino acid signal (Kawai *et al.*, 2011).

### 3. Evaluation of the role of the guanylic nucleotide pool in TORC1 signalling:

Guanylic nucleotides are a class of molecules implied in a wide range of processes including synthesis of nucleic acids, metabolic reactions and signalling. Generally, when bound to GTP, small GTPases are activated and can interact with and signal to downstream effector proteins. When bound to GDP, they remain in an inactive state. It has already been shown that the GTP/GDP ratio rapidly decreases upon diauxic shift, and is even more affected upon starvation. This ratio controls Ras GTPase activity and thus PKA activity (Rudoni *et al.*, 2001). Moreover, it has been shown that in mammals TORC1 acts as an ATP sensor through AMPK activation (Inoki and Guan, 2006; Dennis *et al.*, 2011).

Mycophenolic acid (MPA) inhibits the synthesis of guanylic nucleotides through binding and inhibition of the inosine monophosphate dehydrogenase (Imd2). MPA is a non-competitive and reversible inhibitor of Imd2 (Franklin and Cook, 1969). It affects yeast cell size, DNA content, budding pattern and causes occasional perturbations of actin and microtubule cytoskeletons (Escobar-Henriques, 2001). The purine biosynthesis pathway is tightly linked to and strongly depends on amino acid biosynthesis and amino acid deprivation might affect GTP synthesis. Depletion of GTP decreases proliferation of various cell types (Morath and Zeier, 2003; Yalowitz and Jayaram, 2000), and MPA has already been shown to affect signal transduction pathways in T cell in addition to its inhibitory role on replication (Wilson *et al.*, 1989). As Imd2 catalyses the rate limiting step in GTP biosynthesis, it is very appealing to think that Gtr1 might be a sensor of GTP and, depletion of GTP might be a signal for TORC1 in the control of cell growth.

To test this hypothesis, we took advantage of mycophenolic acid (MPA) to deplete the intracellular pool of GTP. MPA slightly inhibited growth of yeast cells on plates. However, over-expression of the GTP-bound form of Gtr1 could not rescue the sensitivity of yeast cells (Figure 19A). To confirm that the effects of MPA on growth were dependent on TORC1, we assessed the level of phosphorylation of Sch9. MPA strongly affected the phosphorylation status of Sch9 but, again, expression of $Gtr1^{GTP}$ could not overcome the effects of the drug (Figure 19B). Moreover, this effect was specific with respect to the inhibition of Imd2 activity, as addition of guanine could suppress the corresponding inhibition (Figure 19B, lower panel).

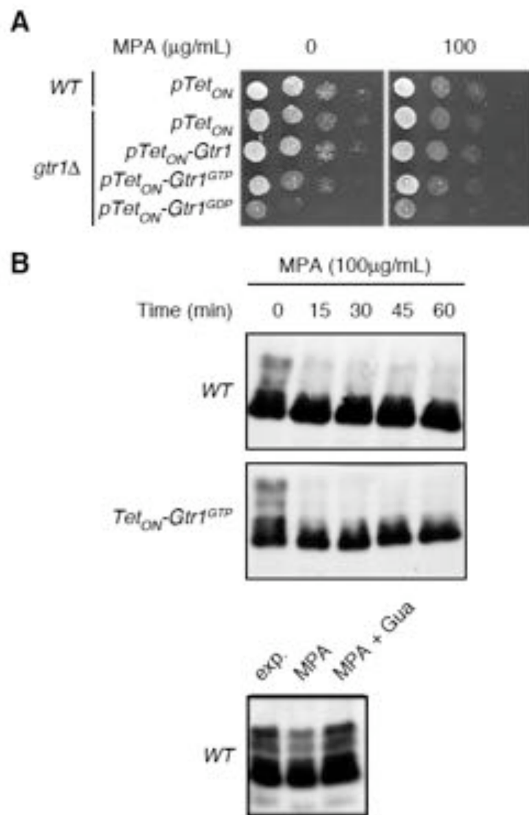



**Figure 19: Mycophenolic acid (MPA) inhibits growth and TORC1 activity independently of the EGOC**

(A): MPA slightly inhibits growth of WT and gtr1Δ cells expressing the different alleles of Gtr1. Serial 10-fold dilutions of WT and mutants cells were spotted on SD plates containing the indicated concentrations of MPA and grown for two days.

(B): WT cells expressing vector (WT) or Gtr1$^{GTP}$ from the doxycycline inducible promoter (Tet$_{ON}$-Gtr1$^{GTP}$) were grown to mid-log phase in SD medium in the presence of doxycycline (5 µg/mL) and treated with MPA (100 µg/mL). TORC1 activity was monitored over time by assessment of Sch9 phosphorylation status. Addition of guanine (0.3 mM) together with MPA (100 µg/mL) for 30 min was used as a control for the specifity of the drug (lower panel).

It is remarkable that GTP depletion affects very strongly TORC1 activity, although this effect is independent of the EGOC. This result underlines one more time that TORC1 regulation is complex. It is not clear whether GTP is a direct signal for TORC1, or if GTP depletion affects TORC1 activity through an indirect pathway. Notably, guanine nucleotide depletion affects many cellular processes among which translation, Ras signalling and DNA replication (for review see (Ljungdahl and Daignan-Fornier, 2012)).

# III. Discussion

By targeting different steps in the TCA cycle, we have seen that the production of glutamine is required for TORC1 signalling, as inhibition of the glutamine synthetase Gln1 mimics the effects of glutamine depletion, and completely abolishes TORC1-dependent Sch9 phosphorylation. Moreover, we have shown that inhibition of the production of GTP also inhibits TORC1 signalling. However, none of the drugs tested act via the EGOC to inhibit TORC1 activity, as expression of GTP-restricted Gtr1 cannot prevent the TORC1 inactivation mediated by these drugs.

In the course of this study, the role of glutamine in TORC1 activation has been revisited in higher eukaryotes, and it has been shown in mammals that glutamine is necessary for full activation of mTORC1 following leucine stimulation. Precisely, glutamine uptake by the $Na^+$-dependent high affinity transporter SLC1A5 is a pre-requisite for the antiport of leucine by the branched-chain amino acid transporter SLC7A5 (Nicklin *et al.*, 2009). However, it is unlikely that this process exists in yeast, as SLC1A5 does not seem to have a yeast homologue and SLC7A5 shows little homology with any of the yeast amino acid permeases. Moreover, no amino acid antiporter at the plasma membrane has been described in *S. cerevisiae* in which amino acid import by a wide variety of permeases in the cytoplasm is rather direct (De Craene *et al.*, 2001; MacGurn *et al.*, 2011; O'Donnell *et al.*, 2010; Schmidt *et al.*, 1998).

We could show that TORC1 is sensitive to inhibition of the purine biosynthesis, and more particularly to GTP depletion, although this signal is not conveyed by the EGOC. This observation is interesting regarding the fact that, in mammals, transcription of the inosine monophosphate dehydrogenase (IMPDH) genes is linked to cell proliferation and leukemic cells have increased IMPDH enzymatic activity (Konno *et al.*, 1991). Inhibition of the IMPDH leads to a cell cycle arrest in G1 (Messina *et al.*, 2004), a phenotype observed upon TORC1 inhibition. Moreover, the GTP/GDP balance is decreased when cells enter into quiescence (Rudoni *et al.*, 2001). Thus, it is appealing to imagine that the intracellular guanylic nucleotide pools might sustain growth, partly through TORC1.

# - Chapter III -

# Identification of New Regulators of the EGOC

# I. Introduction

In yeast, TORC1 remains associated with the vacuolar rim and studies of mTORC1 localisation show that, under physiological conditions, it is constitutively associated with lysosomes (Korolchuk *et al.*, 2011). How EGOC/Rag-Ragulator complex senses amino acids and whether it acts as a scaffold to localise TORC1 remains to be addressed. Additionally, the intra-cellular sensor of amino acids remains to be discovered, as it seems unlikely that the GTPases directly bind amino acids.

Gtr1/RagA/B, in its GTP-bound state, is required for the interaction with Kog1/Raptor and stimulation of TORC1; however, additional experiments are required to decipher the molecular mechanism of Gtr1 activation. Moreover, the exact role of Gtr2/RagC/D is nebulous and the mechanism of regulation of both GTPases is a key issue that has to be addressed. Notably, we know that Vam6 operates as a GEF on Gtr1 but its corresponding GAP is still unknown as are the GAP and GEF of Gtr2. Identification of these regulatory proteins is crucial to fully understand the regulation of the EGO complex and consequently TORC1.

In order to identify possible interactors of Gtr1, we decided to use two different large-scale approaches:

- a classical LexA-based Yeast Two Hybrid screen (YTH) using Gtr1 as the bait protein and a genomic library expressing the corresponding preys.
- a Tandem Affinity Purification (TAP) of Gtr1 followed by tandem mass spectrometry analysis (MS/MS) of the co-precipitating partners.

# II. Results

## 1. Identification of new Gtr1 interactors using a Yeast Two-Hybrid system

### *i Analysis of the potential candidates*

The screen was performed essentially as described elsewhere (Cagney *et al.*, 2000) and see materials and methods) with Gtr1 fused to the LexA DNA-binding domain (DBD). Following transformation with the genomic DNA library, positive clones were selected based on their ability to activate the *LEU2* and *LacZ* reporter genes, as assessed by monitoring growth on a medium lacking leucine and by filter assays with the chromogenic substrate X-Gal, respectively. Over 3 days of incubation, we selected 496 clones able to grow in the absence of leucine, and this number was narrowed down to 372 following X-Gal filter assays. After plasmid recovery and retransformation, only 26 clones could be reconfirmed by liquid β-galactosidase assay, indicating that the screen generated a surprisingly high number of false positives. The 26 plasmids harboring the DNA fragments fused to VP16 AD were subsequently sequenced, and intriguingly, most of the retrieved sequences corresponded to 2μ plasmid sequences or mapped to the non-coding strand of the gene. The final results (summarized in Table 4) show that the screen was not saturated (only 3 clones were recovered more than once) and yielded none of the known Gtr1 interactors.

| Prey | β-galactosidase activity (M.U.) | Number of clones | Fragment of the protein | Gene description |
|---|---|---|---|---|
| Brf1 | 456 | 2 | 230 to 479 (/596) | Subunit of the TFIIIB transcription initiation factor |
| Nqm1 | 472 | 2 | 204 to 333 (/333) | Transaldolase |
| Cym1 | 287 | 2 | 272 to 517 (/989) | Lysine specific metalloprotease of the mitochondria |
| Sac3 | 161 | 1 | 263 to 546 (/1301) | Nuclear pore protein of the TREXII complex |
| Rpl25 | 2432 | 1 | 5 to 86 (/142) | Ribosomal protein of large (60S) subunit |

**Table 4: Gtr1 potential interactors found by two-hybrid screening**
β-galactosidase activities were measured in three independent experiments after growth for 6 hr at 30°C in SGal/Raf medium. The average values (in Miller units, M.U.) are shown.

In addition, several of the positive clones are unlikely *bona fide* Gtr1 interactors. For instance, the mitochondrial localisation of Cym1 does not fit with the Gtr1 vacuolar membrane localisation (Huh *et al.*, 2003), as is the case for the nuclear pore localisation of Sac3 (Lei *et al.*, 2003). Nqm1 is a transaldolase whose expression is induced during the diauxic-shift when Gtr1 is

supposed to be inactive (Dasgupta *et al.*, 2002). The ribosomal protein Rpl25 is required for assembly and for processing of the large ribosomal subunit, and it also plays a post-translational role, notably by recruiting chaperones (Dalley *et al.*, 2008). As such Rpl25 could interact non-specifically with Gtr1. Finally, We decided to focus on Brf1 as a possible interactor of Gtr1.

*ii The TFIIIB-related transcription factor Brf1 is a potential interactor of Gtr1*

It has been shown that a fraction of both Gtr1 and TORC1 localise in the nucleus (Li *et al.*, 2006; Nakashima *et al.*, 1996; Wei *et al.*, 2009). The exact role of Gtr1 in the nucleus is unclear but it has been proposed to play a role in the Ran GTPase cycle (Nakashima *et al.*, 1996; Nakashima *et al.*, 1999). As *gtr1Δ* cells show decreased levels of rRNAs, Gtr1 has also been implicated in RNA Pol I and Pol III transcription (Todaka *et al.*, 2005). Notably, TORC1 regulates RNA polymerases (Laferte *et al.*, 2006; Mayer *et al.*, 2004) and *gtr1* mutants display a low TORC1 activity. Thus, the observed effect of *gtr1* deletion on RNA Pol I and Pol III transcripts may rather be indirect. As a consequence, it may be possible that the interaction observed between Gtr1 and Brf1 is indirect and mediated by the fraction of TORC1 in the nucleus. Of note, the presence of Gtr1 in the nucleus indicates that it may also activate nuclear TORC1.

As a first step, we tested if the interaction between Gtr1 and Brf1 was dependent on the nucleotide loading status of Gtr1 as it is the case for Kog1-Gtr1 and Tco89-Gtr1 interactions (Binda *et al.*, 2009). Thus, the full-length ORF of *BRF1* was cloned into the prey plasmid and transformed together with a plasmid expressing either Gtr1-DBD or its alleles Gtr1-Q65L or S20L (GTP and GDP-restricted, respectively) into the EGY48 yeast two-hybrid strain. Yeast cells were grown to mid-log phase in YPG-HUT medium and the β-galactosidase activity was measured. The couples Gtr1-DBD/Gtr2-AD and Gtr1-DBD/AD served as positive and negative controls, respectively. As expected, Gtr1 and Gtr2 specifically interacted with each other. Furthermore, the interaction between Gtr1 and the full-length Brf1 protein was confirmed and this interaction was dependent on the nucleotide loading status of Gtr1 since the GDP-restricted allele of Gtr1 did not interact with Brf1. Brf1-DBD form is functional, as it interacts with Gtr1, and does not activate the reporters, because it yields only background activity when combined with AD-Gtr1$^{GDP}$ (Table 5).

The different alleles of Gtr1 were expressed to the same levels (Figure 20). This observation also rules out the possibility that Brf1 activates transcription of the *lacZ* gene on its own. Indeed, Brf1 could have induced a positive response due to its intrinsic function as a transcription factor. The interaction between Gtr1 and Brf1 is not as strong as the Gtr1-Gtr2 interaction but it is quite stable suggesting that Brf1 is probably a transient partner of Gtr1.

| AD fusion | β-galactosidase activity with DBD fusion (M.U.) | | |
|---|---|---|---|
| | BRF1 | GTR2 | Empty vecor |
| GTR1 | **456.6** | **1387** | 0.7 |
| GTR1-S20L | 2.4 | n.d. | n.d. |
| GTR1-Q65L | **289.3** | n.d. | n.d. |

**Table 5: Gtr1 interacts with Brf1 preferentially in its GTP-bound form**
EGY48 yeast cells expressing WT *GTR1* or the indicated alleles of *gtr1* from the bait vector, and the indicated gene from the prey vector, were grown to mid-log phase in YPG-HUT and assayed for β-galactosidase activity. The results represent the mean and SD of three independent clones and are expressed in Miller units (M.U.). n.d.: not determined.

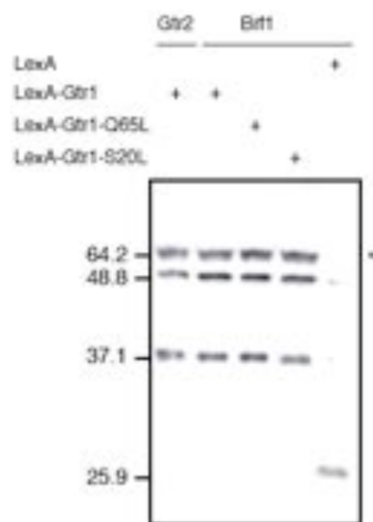



**Figure 20: the expression levels of the different alleles of LexA-Gtr1 are similar.**
EGY48 yeast cells expressing WT *GTR1* or the indicated alleles of *gtr1* from the bait vector, and the indicated gene from the prey vector, were grown to mid-log phase in YPG-HUT. Following post-alkaline extraction, proteins were separated on a 7.5% SDS-PAGE and analyzed by anti-LexA western blotting. The asterisk (*) indicates the band corresponding to LexA-Gtr1.

Brf1 is a phosphoprotein and its phosphorylation status determines its recruitment to promoters (Fairley *et al.*, 2012; Fairley *et al.*, 2003; Felton-Edkins *et al.*, 2003). Moreover, it has recently been demonstrated that Sch9, a direct substrate of TORC1, phosphorylates Maf1, a conserved negative regulator of Pol III transcription (Huber *et al.*, 2009; Pluta *et al.*, 2001; Upadhya *et al.*, 2002). We therefore tested if Brf1 could be a substrate for TORC1 or Sch9 *in vitro*. TORC1 was immuno-precipitated from a yeast strain expressing a TAP-tagged version of Tco89 (Urban *et al.*, 2007) and mixed with recombinant GST-tagged Brf1 purified from bacteria. Purified proteins were mixed and incubated in the presence of [$\gamma$–$^{33}$P]ATP to test the kinase activity of TORC1 toward Brf1. GST-tagged Fpr1, expressed and purified from bacteria, served as a negative control once mixed with rapamycin. The complete TOR Complex 1 was immunoprecipitated and, except Lst8, each partner of the complex could be identified on the silver stained gel as judged from the corresponding sizes of the co-precipitating bands. A positive control (*e.g.* Sch9) to monitor TORC1 activity is missing and, although Brf1 does not seem to be phosphorylated *in vitro* by TORC1, we cannot completely exclude this possibility (Figure 21).

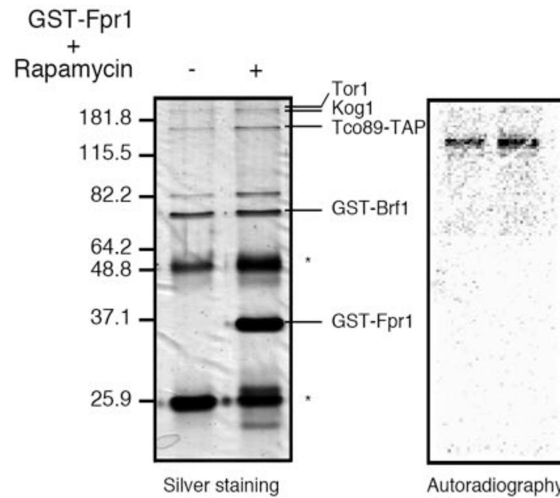



**Figure 21: TORC1 does not phosphorylate Brf1 *in vitro***
TORC1 was purified from lysates of yeast cells exponentially growing in SD and expressing an endogenous C-terminally TAP-tagged version of Tco89 by immunoprecipitation with IgG sepharose beads. GST-Brf1 and GST-Fpr1 were purified from bacteria as described previously (Binda *et al.*, 2009). Following kinase assay, the proteins were resolved by SDS-PAGE and the gels were silver stained (Gromova and Celis, 2006) and exposed for autoradiography. The asterisks (*) indicate non-specific IgG bands.

In parallel, Sch9-HA and a kinase dead version of the enzyme (Sch9-K441A-HA) were purified from yeast and mixed with recombinant GST-Brf1. Although the purification of Sch9 contained additional bands, the kinase and its inactive variant were efficiently immunoprecipitated (Figure 22 and data not shown). The extra bands observed could be degradation products or proteins binding non-specifically. Furthermore, we could observe bands in the very high molecular weight region that could correspond to TORC1 components. As for the previous kinase assay, a positive control (*e.g.* Maf1 or Rps6) should have been included to validate our observation that Sch9 does not phosphorylate Brf1 *in vitro* (Figure 22).

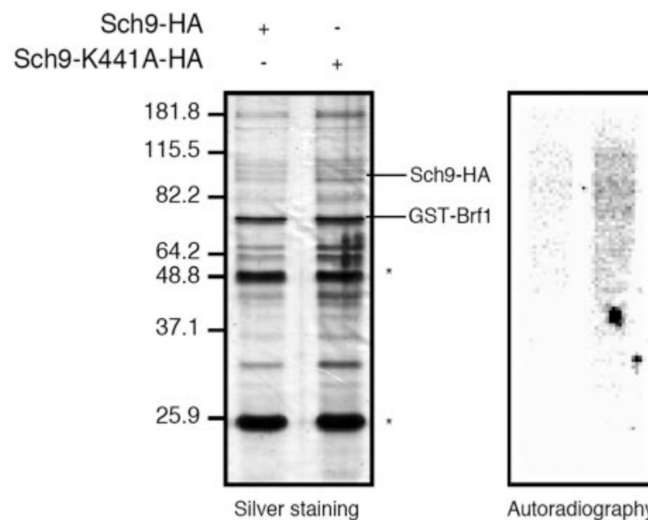



**Figure 22: Sch9 does not phosphorylate Brf1 *in vitro***
Sch9 or Sch9-K441A-HA was endogenously expressed from a plasmid in WT cells exponentially growing in SD, and immunoprecipitated from lysates with 12CA5 mouse anti-HA antibodies. Following kinase assay, the proteins were resolved by SDS-PAGE and the gels were silver stained (Gromova and Celis, 2006) and exposed for autoradiography. The asterisks (*) indicate non-specific IgG bands.

Ribosome biogenesis is a highly energy consuming process (Warner, 1999) that needs tight regulation. While TORC1 and its downstream effector Sch9 have clearly been shown to directly control RNA Pol III transcription through Maf1, we did not observe a biochemical link between the TORC1 signalling pathway and the TFIIIB transcription factor Brf1. Nevertheless, based on our rather preliminary data, we cannot definitely exclude the possibility that EGOC-TORC1 controls RNA Pol III transcription impinging on Brf1.

## 2. Identification of new Gtr1 interactors by Tandem Affinity Purification/Mass spectrometry (TAP/MS)

The tandem-affinity purification method is the method of choice to isolate protein complexes and identify relevant physiological interacting partners of a protein of interest (Rigaut *et al.*, 1999). We used this approach to identify physical partners of Gtr1 *in vivo*. Accordingly, leucine auxotrophic yeast cells expressing Gtr1-TAP were grown to mid log-phase in SD medium supplemented with the required amino acids, and half of the culture was submitted to leucine deprivation for 1 hour. Cells were harvested and processed following the TAP purification protocol. This approach allowed us to identify amino acyl-tRNA synthetases (AARSs) as potential Gtr1 partners. Preceding the description of the results, I will therefore briefly introduce in the following paragraphs some general features of AARSs that should facilitate the appreciation of the experimental design.

### *i Amino acyl tRNA synthetases*

#### a. General remarks

Aminoacyl-tRNA synthetases are a class of enzymes that catalyse the esterification of a specific amino acid to one of its compatible cognate tRNAs. The reaction implies activation of the amino acid with an ATP to form an aminoacyl-adenylate that is attached in a second reaction to the tRNA. The aminoacylated-tRNA resulting from this charging reaction is used by the ribosome during the process of translation to transfer the amino acid, according to the genetic code, onto the newly synthesised peptide chain. The uncharged tRNA can be recycled back for a new cycle of charging.

Based on their structural features, AARSs can be separated into two classes. Class I enzymes have a catalytic domain that adopts a Rossmann-fold structure (Rossmann *et al.*, 1974) whereas class II enzymes have an antiparallel $\beta$-fold flanked by $\alpha$-helices (Cusack *et al.*, 1990; Rould *et al.*, 1989; Ruff *et al.*, 1991). Class I AARSs are monomeric enzymes characterized by the two highly conserved HIGH and KMSKS motifs, which are responsible for the substrate recognition. Additionally, they attach the amino acid on the 2'OH group of the final ribose at the 3'-end of the tRNA. Class II AARSs are characterized by three highly conserved motifs. Motif 1 allows dimerization, while the two other motifs are responsible for substrate recognition and catalysis of the aminoacylation of the tRNA. In class II enzymes, the amino acid is attached on the 3'OH of the final ribose at the 3'-end of the tRNA (Sankaranarayanan and Moras, 2001). Intriguingly, synthetases from the two classes bind to different faces of the tRNA molecule. In addition, the two classes can be subdivided into three groups depending on additional structural features (Table 6) (Delarue and Moras, 1993).

| | Class I | Class II |
|---|---|---|
| Group a | | |
| | CysRS | GlyRS |
| | ValRS* | AlaRS* |
| | LeuRS* | SerRS |
| | IleRS* | ProRS* |
| | MetRS* | ThrRS* |
| | ArgRS | HisRS |
| Group b | | |
| | GlnRS | AspRS |
| | GluRS | AsnRS |
| | LysRS | LysRS |
| Group c | | |
| | TyrRS | PheRS* |
| | TrpRS | GlyRS |

**Table 6: Classification of aminoacyl-tRNA synthetases according to their structural and functional organization**
The enzymes with an asterisk (*) were shown to possess an editing activity.

Aminoacyl-tRNA synthetases ensure the fidelity of the translation process by accurately discriminating a specific amino acid out of the 20 amino acids that occur in proteins, and the few amino acids that do not occur in proteins. In addition, AARSs specifically discriminate cognate tRNAs out of the twenty families of tRNA (Jakubowski and Goldman, 1992). While recognition of the tRNA is facilitated by its large structure, amino acid discrimination is much more complex given the resemblances between amino acids with similar side chains. As a consequence, the error rate in tRNA selection is about $10^{-6}$ whereas the estimated error rate for amino acid selection is between $10^{-4}$ to $10^{-5}$ (Loftfield and Vanderjagt, 1972). To ensure proper charging of their cognate tRNAs, some AARSs have evolved a proofreading or editing activity. In the following paragraph I will focus on the editing mechanism by class I enzymes.

b. Editing by Class I amino acyl-tRNA synthetase

Among the ten class I enzymes, four were shown to display an editing activity (LeuRS, IleRS, MetRS and ValRS), which occurs within the active site by hydrolysis of the aminoacyl adenylate (pre-transfer), or at a separate site called the editing (or CP1) site to deacylate the mischarged aminoacyl-tRNA (post-transfer). All four enzymes use both editing mechanisms with some preferences for one or the other. Crystallographic and biochemical *in vitro* studies have shown that ValRS and LeuRS would rely more on the post-transfer editing mechanism, whereas IleRS and MetRS use both pre- and post-transfer (Chen *et al.*, 2011; Fersht, 1977; Fersht and Kaethner, 1976; Jakubowski, 2011; Tukalo *et al.*, 2005). This difference may depend on substrate levels or on the nature of the noncognate amino acid (Boniecki *et al.*, 2008; Chen *et al.*, 2011; Jakubowski, 2011).

The pre-transfer mechanism hydrolyses the aminoacyl adenylate molecule either in a tRNA-dependent or independent fashion, the former one being the most common pre-transfer editing pathway (Jakubowski, 2011). During post-transfer editing, the mischarged tRNA is translocated from

the active site to the editing site for deacylation (Ling *et al.*, 2009). It has notably been shown for the LeuRS that the translocation of the mischarged 3'-end of the $tRNA^{leu}$ requires rotation of the editing domain (Tukalo *et al.*, 2005). In the editing domain, a highly conserved region containing the catalytic residues, and an essential aspartic acid residue, hydrolyses the mischarged $tRNA^{leu}$ (Yao *et al.*, 2008b). In *S. cerevisiae*, mutation of the aspartic acid residue 419 to alanine results in an editing defective LeuRS unable to grow on the leucine analog norvaline (Rock *et al.*, 2007; Yao *et al.*, 2008b).

c. Possible implication of tRNA synthetases in TORC1 signalling

A central question in the control of cell growth by amino acids concerns the sensor to which amino acids bind. Aminoacyl-tRNA synthetases were proposed to perform such a function because of their amino acid binding capacity, and several groups have studied the possibility that AARSs signal to TORC1. However, the results are contradictory and, while some studies tend to show an implication of AARSs in TORC1 signalling, others do not. This could be due to the fact that the experiments were conducted in different organisms or with different cell lines, amino acid analogues or genetic tools. For instance, in Jurkat cells, amino alcohols, which are supposed to competitively inhibit AARSs, inhibit S6K activity, indicating that tRNA aminoacylation might be required for mTORC1 signalling. However, it is not clear whether tRNA charging is affected or not upon amino alcohol treatment of Jurkat cells (Iiboshi *et al.*, 1999). In contrast, Lynch *et al.* showed that leucinol is neither inhibiting nor activating TORC1 activity in freshly isolated adipocytes from rats. In addition, the authors exclude the possibility that leucyl-tRNA or LeuRS signal to mTORC1, a conclusion that is based on their pharmacological studies of different leucine analogues (Lynch *et al.*, 2000). In contrast to the two previous studies, Christie *et al.* demonstrated that in *Xenopus laevis* oocytes, leucinol and other leucine analogues are very potent activators of TORC1. As a consequence, the authors propose that amino acids might be sensed by an amino acid receptor, or by an indirect mechanism reflecting amino acids concentrations (Christie *et al.*, 2002). Finally, experiments conducted in Chinese hamster ovary cells indicated that neither accumulation of uncharged tRNAs nor LeuRS charging activity are required for leucine sensing and signalling to TORC1. This was shown with a thermo-sensitive allele of LeuRS, which, upon inactivation, does not change TORC1 signalling, while it is not functional anymore for the proper charging of $tRNA^{leu}$ (Wang *et al.*, 2008). However, this study could not definitely exclude the possibility that LeuRS signals to TORC1. Thus, taken together, the literature on the potential role of AARSs in mediating an amino acid signal towards TORC1 does no convey a clear picture.

*ii 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 signalling 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 co-precipitating 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 7).

| 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 |

**Table 7: Proteins Identified in Gtr1-TAP Pull-Down Experiments**
[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.

This finding, which we independently confirmed in co-precipitation assays using an endogenously tagged version of Cdc60 (Figure 23A, B and C), suggests that the LeuRS Cdc60 may play a role in signalling leucine availability to Gtr1-TORC1.

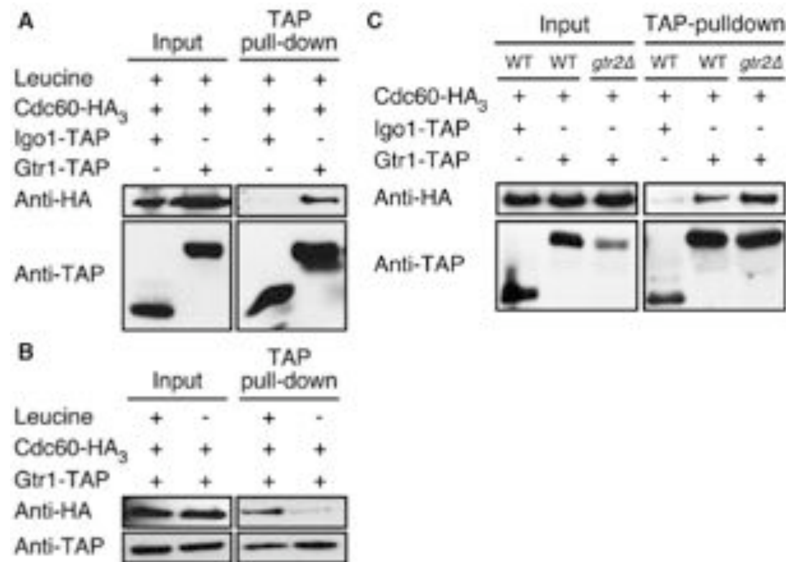



**Figure 23: LeuRS Cdc60 Physically Interacts With the TORC1 Regulator Gtr1 in a Leucine-Dependent Manner**

(A, 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.

(C) 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.

Of note, eukaryotic LeuRSs exhibit two functionally separate activities, namely an essential $tRNA^{Leu}$ aminoacylation activity and an amino acid proofreading (editing) activity, which 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 24A) that is defective in $tRNA^{Leu}$ aminoacylation and therefore accumulates uncharged $tRNA^{Leu}$ at the restrictive temperature (Hohmann and Thevelein, 1992). In control experiments, phosphorylation of the eukaryotic translation initiation factor $2\alpha$ (eIF$2\alpha$) at $Ser^{51}$ - a sensitive indicator of the presence of uncharged tRNAs ($tRNA^{Leu}$) that stimulate the eIF$2\alpha$-kinase Gcn2 (Hinnebusch, 2005) - strongly increased in *cdc60*$^{ts}$, but not in wild-type cells, when incubated for 1 or 2 hrs at 37°C (Figure 24B). Under the same conditions, temperature-inactivation of $Cdc60^{ts}$, however, had no significant impact on TORC1 activity, as assessed by monitoring the phosphorylation level of the TORC1 substrate Sch9 (Figure 24B) (Urban *et al.*, 2007). These observations, which 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.

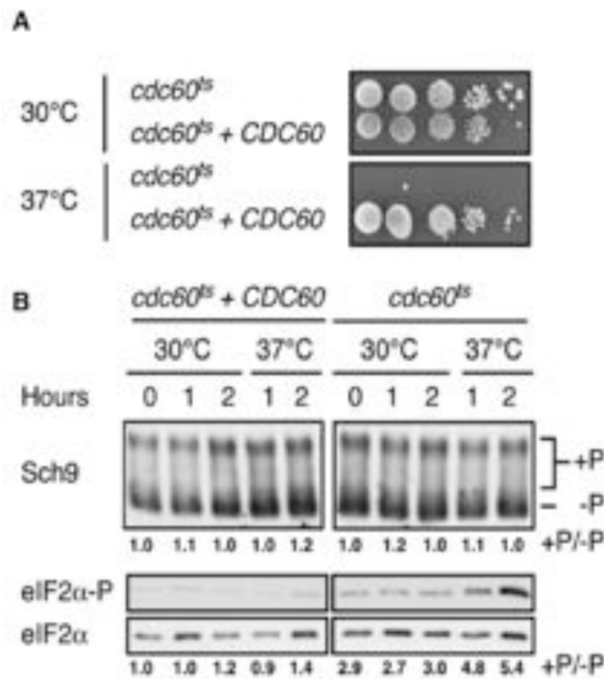



**Figure 24: Cdc60-Mediated tRNA$^{Leu}$ Aminoacylation Is Not Required for Normal TORC1 Activity**

(A) 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.

(B). Immunoblots detecting the extent of Sch9 phosphorylation were used to quantify *in vivo* TORC1 activity (Urban *et al.*, 2007) in exponentially growing wild-type (*cdc60$^{ts}$* harboring a plasmid expressing *CDC60*) and *cdc60$^{ts}$* strains that were grown at the indicated temperatures (upper panel; 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).

### *iii Trapping of tRNALeu Within the LeuRS Editing Site Downregulates EGOC-TORC1 Signalling*

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 25A) (Rock *et al.*, 2007). Surprisingly, DHBB treatment, which did not noticeably alter Gtr1-GFP and Tor1-GFP localisation (Figures 25D-E), resulted in significant downregulation of TORC1 activity in wild-type cells, but not in cells expressing the DHBB-resistant Cdc60$^{D418R}$ variant (Rock *et al.*, 2007; Yao *et al.*, 2008b) (Figures 25B and C). In addition, co-expression 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$^{Leu}$, activation of Gcn2, or inhibition of growth in DHBB-treated cells (Figures 25A, B and C).

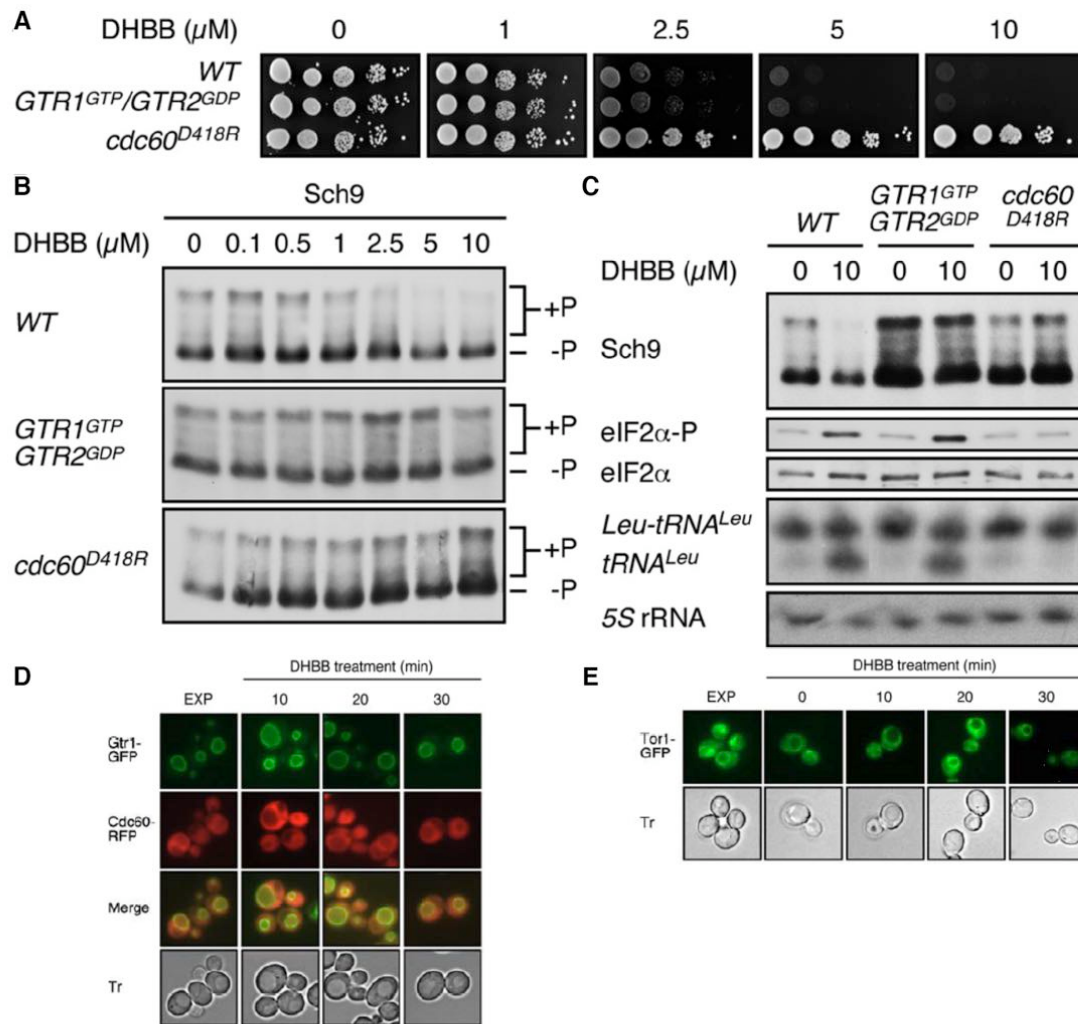



**Figure 25: DHBB-Mediated Trapping of tRNA$^{leu}$ Within the LeuRS Editing Site Downregulates EGOC-TORC1 Signalling without affecting their localisation**
(A) DHBB treatment inhibits growth of wild-type (WT) and Gtr1$^{GTP}$/Gtr2$^{GDP}$-expressing cells, but not of cells expressing the DHBB-resistant Cdc60$^{D418R}$ variant. Serial 10-fold dilutions of cells were spotted on SD plates containing the indicated concentrations of DHBB.
(B, 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.
(C, D) DHBB treatment does not affect the localisation of Gtr1-GFP (C) or Tor1-GFP (D). Gtr1-GFP (C) and Tor1-GFP (D) mainly localize to the vacuolar membrane, while Cdc60-RFP (C) 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 localisation 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.

Moreover, DHBB disrupted, in a concentration-dependent manner, the Gtr1-Cdc60 (and Gtr1$^{GTP}$-Cdc60; Figure 26D), but not the Gtr1-Cdc60$^{D418R}$ interaction (Figures 26A and B). 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 co-precipitates 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 26C).

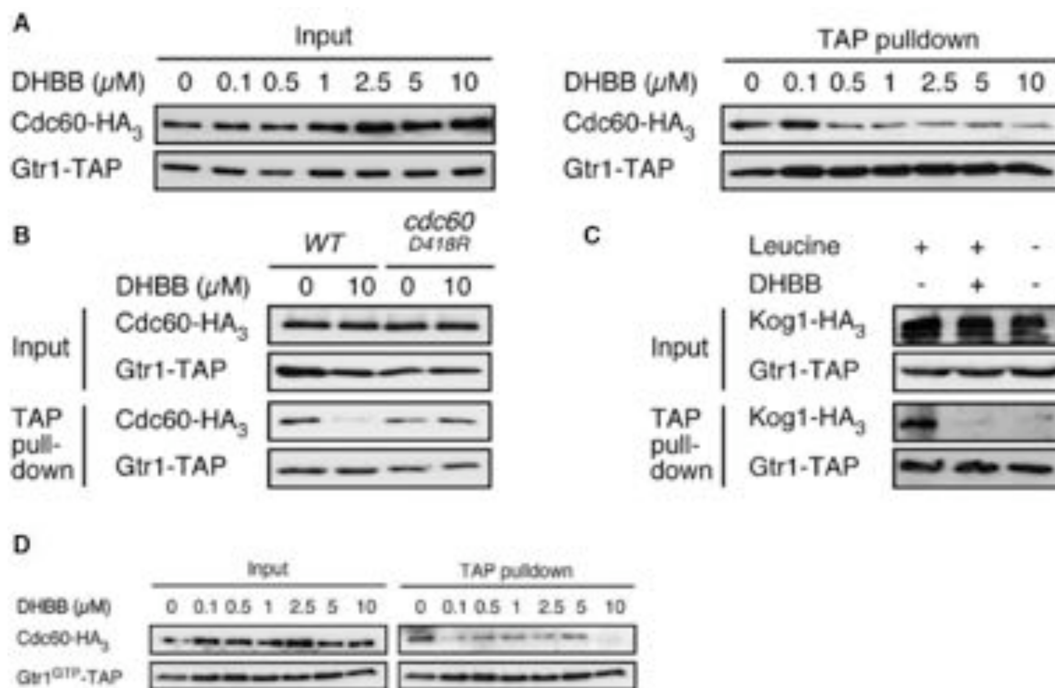



**Figure 26: DHBB-Mediated Trapping of tRNA$^{leu}$ Within the LeuRS Editing Site**
(A, B) DHBB treatment (30 min) disrupts the Cdc60-Gtr1 interaction (A), but not the Cdc60$^{D418R}$-Gtr1 interaction (B), in a concentration-dependent manner.
(C) Gtr1-Kog1 interaction is sensitive to both DHBB treatment 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.
(D) 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.

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 binding the DHBB-tRNA$^{Leu}$ adduct (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.*, 2008b) (Figure 27A), 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, as temperature-inactivation of Cdc60$^{ts}$ significantly protects TORC1 from leucine starvation-induced downregulation (Figure 27B).

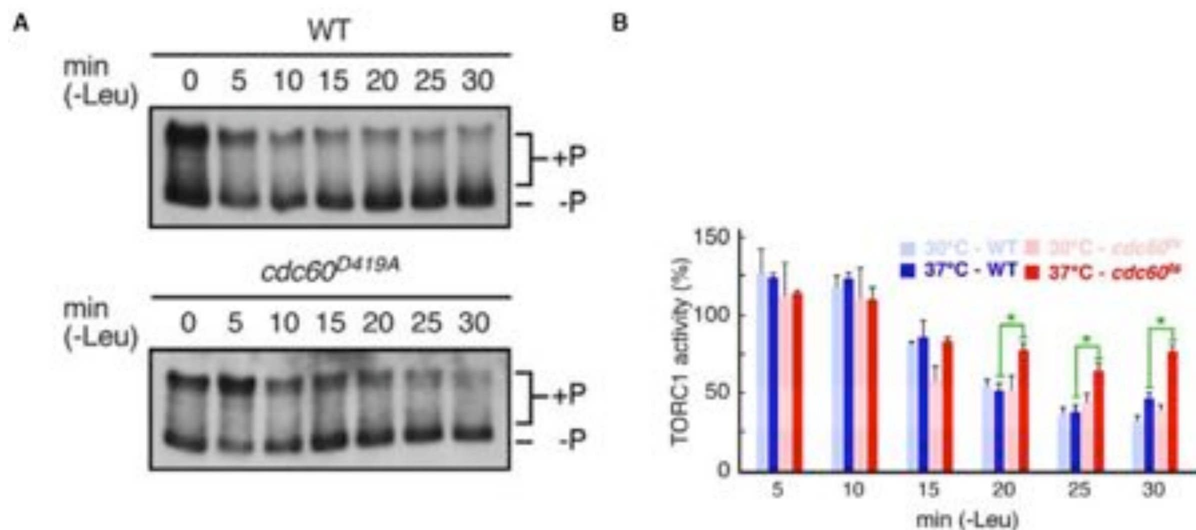



**Figure 27: Physiological Relevance of LeuRS Editing**
(A) Leucine starvation causes TORC1 inactivation in both wild-type and *cdc60*$^{D419A}$ mutant cells. Leucine (*leu2*Δ) auxotrophic wild-type (WT) and *cdc60*$^{D419A}$ mutant cells were grown to exponential phase in medium containing leucine and 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 described previously.
(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 D. 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).

#### *iv The LeuRS Inhibitors Leucinol and Norvaline Oppositely Affect EGOC-TORC1 Signalling*

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), blocks LeuRS-mediated aminoacylation (and growth; Figures 28A and B), and thus impedes LeuRS from engaging in editing activities. Nva, in contrast, 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, which limits growth at higher Nva concentrations (Figure 28A). Consistent with our model and its mode of action towards LeuRS, LeuOH did not cause TORC1 downregulation (Figure 28C). 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 wild-type, but not in *gtr1*Δ cells (Figure 28C; (Binda *et al.*, 2009); and not shown). As expected, neither LeuOH nor leucine was able to re-stimulate TORC1 in the presence of DHBB (Figure 28D). The results with Nva were equally clear: Nva potently inhibited TORC1 in wild-type cells (without increasing the levels of uncharged tRNA$^{Leu}$; Figure 28A) even when applied in concentrations that are sub-inhibitory for growth (Figures 28B and C).

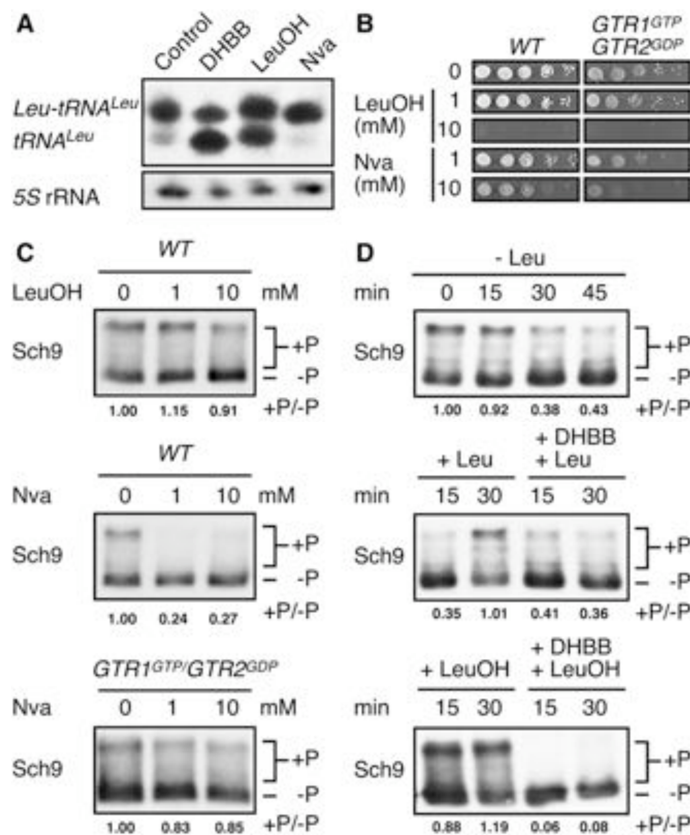



**Figure 28: The LeuRS Inhibitors Leucinol (LeuOH) and Norvaline (Nva), Which Dampen and Stimulate, Respectively, LeuRS Editing, Oppositely Affect EGOC-TORC1 Signalling**

(A) The levels of charged and uncharged $tRNA^{Leu}$ were assayed in wild-type 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) LeuOH and Nva both inhibit growth of wild-type (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 FigureXX.

Importantly, Nva-mediated downregulation of TORC1, but not the observed growth inhibition at higher Nva concentrations, were significantly suppressed by expression of the $Gtr1^{GTP}/Gtr2^{GDP}$-encoding alleles (Figures 28B and 28C). Together with the observations that (i) the addition of a disproportionate quantity of isoleucine causes transient TORC1 inactivation in wild-type cells (Figure 29A), and (ii) LeuRS editing is specifically required for growth under leucine limiting conditions (Figure 29B), these data corroborate a model in which $tRNA^{Leu}$ mischarging following leucine deprivation represents a key signal that impinges on EGOC-TORC1 signalling.

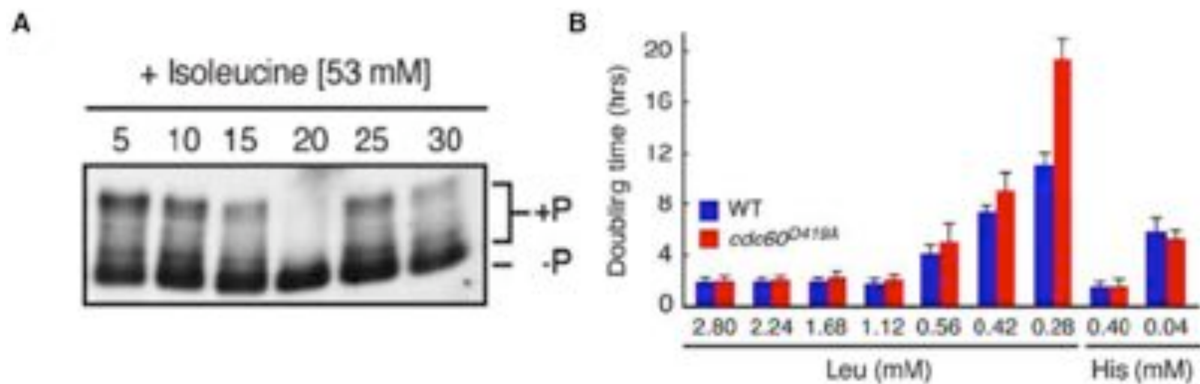



**Figure 29: Physiological Relevance of LeuRS Editing**
(A) Addition of isoleucine in disproportionate quantities causes transient TORC1 inactivation. Wild-type cells were grown (on SD medium) to exponential growth phase and treated with excessive amounts of isoleucine (*i.e.* final concentration of 53 mM). Samples were taken at the times indicated following isoleucine addition and TORC1 activity was assayed as in Figure 1D.
(B) 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.

### *v Mutation of Ser414 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-signalling from LeuRS-tRNA$^{Leu}$ charging. Conceivably, corresponding Cdc60 variants may grant a yet elusive GTPase activating protein access to Gtr1 and thus provoke 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 *gtr1*Δ, caused no obvious growth defect *per se*, but rendered cells defective for growth in the presence of low doses of rapamycin (Figure 30A and B).

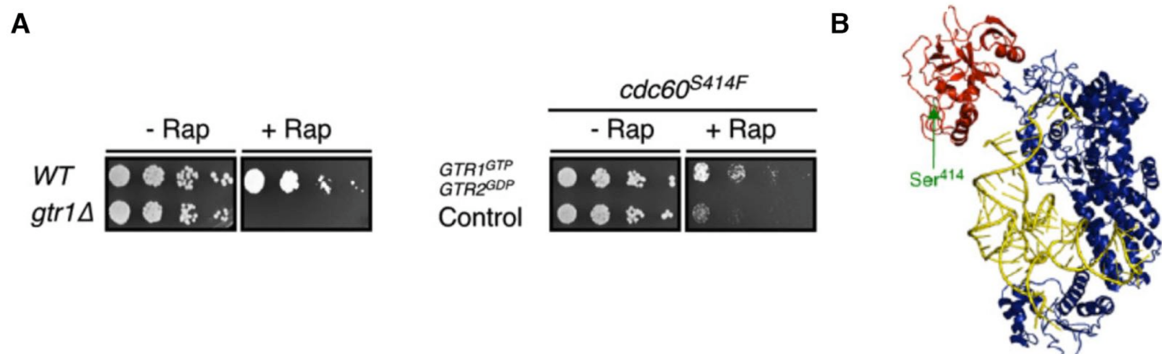



**Figure 30: Mutation of Ser$^{414}$ to Phe Within the CP1 Domain of Cdc60 confers rapamycin sensitivity on plate**
(A) Serial 10-fold dilutions of cells with the indicated genotypes were spotted and grown on either YPD control plates (-Rap), or plates containing low levels (5 ng ml$^{-1}$) of rapamycin (+Rap).
(B) 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).

Since the rapamycin-sensitivity of *cdc60*$^{S414F}$ cells could be suppressed by the expression of the Gtr1$^{GTP}$/Gtr2$^{GDP}$-encoding alleles (Figure 30A), we then used two-hybrid and co-immunoprecipitation (co-IP) 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 31A), but also that the specific Ser$^{414}$ to Phe mutation within this domain abolished the CP1$^{Cdc60}$-Gtr1 interaction (Figures 31A and B).

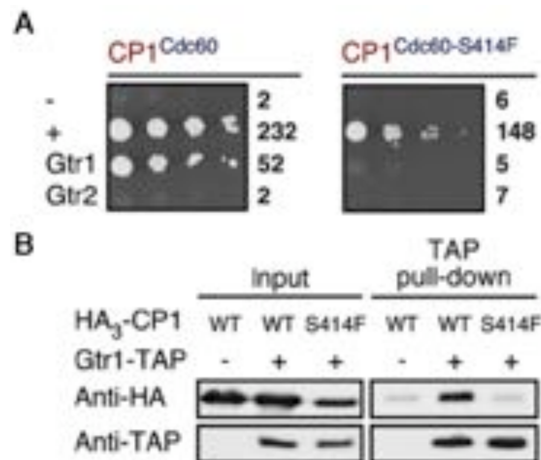



**Figure 31: Mutation of Ser$^{414}$ to Phe Within the CP1 Domain of Cdc60 Disrupts Its Interaction With Gtr1**

(A) 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.

(C) $HA_3$-Cdc60$^{CP1}$ ($HA_3$-CP1; WT), but not $HA_3$-Cdc60$^{CP1\text{-}S414F}$ ($HA_3$-CP1; S414F), co-precipitates with Gtr1-TAP. Cells were harvested in exponential growth phase and pulldown experiments were carried out as in Figure 1B.

Finally, in agreement with a model in which Cdc60 protects Gtr1 from a negative regulator, overproduction of CP1$^{Cdc60}$, but not of CP1$^{Cdc60\text{-}S414F}$, significantly protected TORC1 from inactivation during leucine starvation (Figure 32).

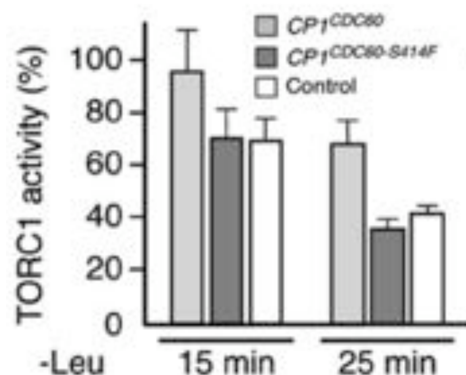



**Figure 32: Overexpression (from the *Tet$_{ON}$* promoter) of *CP1*$^{CDC60}$, but not of *CP1*$^{CDC60\text{-}S414F}$ partially protects TORC1 from inactivation during leucine starvation.**

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 (for details about quantification refer to FigureXX)

In conclusion, LeuRS binds the TORC1-regulator Gtr1 via its CP1 editing domain, which is necessary and sufficient to mediate leucine signalling to TORC1 (Figure 33). Of note, comprehensive analyses of amino acid composition in eukaryotic genomes uncovered that leucine represents the most frequently used amino acid (Echols N. *et al.*, 2002), which, together with the fact that the LeuRS Cdc60 represents the most abundant aminoacyl-tRNA transferase (Ghaemmaghami *et al.*, 2003), also provides a rationale for the preeminent effect of leucine in TORC1 regulation. Since 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 signalling.

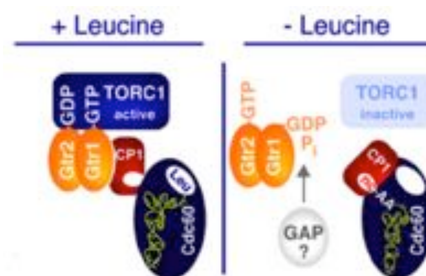



**Figure 33: Model for the role of LeuRS Cdc60-mediated editing in Gtr1-TORC1 signalling. Nc-AA, non-cognate amino acid. For details see the text.**

*vi Implication of additional tRNA synthetases in TORC1 signalling:*

Apart from the leucyl tRNA synthetase, the TAP/MS data indicated that another branched-chain amino acyl-tRNA synthetase, the valyl-tRNA synthetase Vas1, interacted with Gtr1. Interestingly, together with the isoleucyl-tRNA synthetase, these enzymes also belong to the class I of tRNA synthetases, and they both harbor an editing site similar to the one of Cdc60 (PDB accession numbers: 1FFY and 1IYW). Interaction of their CP1 domain with Gtr1 was therefore tested using the split-ubiquitin yeast two-hybrid system. As expected, the CP1 domain of both valyl- and isoleucyl-tRNA ligases interacted specifically with Gtr1 and induced the expression of the two reporter genes (Figure 34A and B). The pattern of growth does not follow the intensity of the interaction seen by β-galactosidase activity measurement, but cells are not in the same physiological state between the two tests (*i.e.* stationary *vs.* exponential, respectively). Following the results of the β-galactosidase assay in which cells are all exponentially growing and in which the strength of the interaction can be roughly estimated, we can see that the CP1 of Cdc60 is the best interactor followed by the Vas1 and Ils1 editing domains. As is obvious in the ClustalW2 alignment of the catalytic region of the three enzymes, the serine 414 of Cdc60 is not perfectly conserved (Figure 34C)(Larkin *et al.*, 2007). Nonetheless, the corresponding amino acid is an alanine that displays equivalent chemical and steric properties. Therefore, we mutated this residue in both isoleucyl- and valyl-tRNA synthetases and assessed the interaction between Gtr1 and these mutant domains by two-hybrid. Unfortunately, these mutations did not prevent the interactions observed previously (data not shown).

| Nub fusion | β-galactosidase activity with Cub-VP16 fusion | |
|---|---|---|
| | GTR1 | GTR2 |
| $CP1^{Cdc60}$ | 52.5 | 2.2 |
| $CP1^{Ils1}$ | 27.5 | 4.0 |
| $CP1^{Vas1}$ | 15.5 | 0.7 |

**Figure 34: Gtr1 specifically interacts with the CP1 domain of the isoleucyl- and valyl-tRNA synthetases in a split-ubiquitin yeast two-hybrid assay**

(A and B) Interactions were assayed by monitoring the growth of NMY51 yeast cells expressing Nub-Gtr1 or -Gtr2 together with the indicated Cub-CP1 (Cdc60, Ils1 or Vas1), on a medium lacking adenine (A) or by liquid β-galactosidase assay (B). pDL2-Alg5 vector served as a negative (-) control (A).

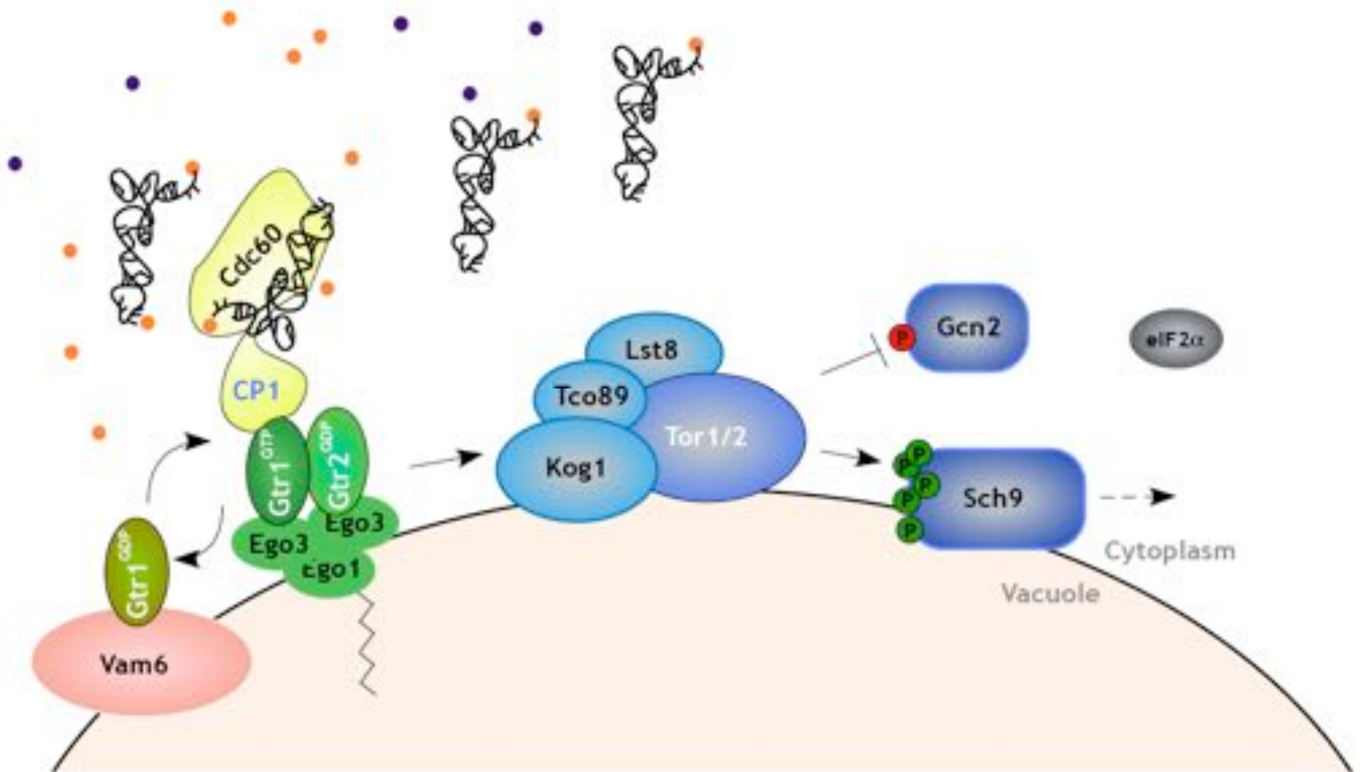
(C) ClustalW2 alignment of the catalytic region within the CP1 domain of LeuRS (Cdc60), ValRS (Vas1) and IleRS (Ils1) from *Saccharomyces cerevisiae*. Cdc60-S414 and the corresponding residues on Vas1 and Ils1 are black boxed. Asterisks (*) indicate fully conserved residues, colon (:) conservation between groups of strongly similar properties and period (.) conservation between groups of weakly similar properties. Small or hydrophobic residues (AVFPMILW) are displayed in red, acidic residues (DE) in blue, basic residues except histidine (RK) in pink, hydroxylated, sulfhydrylated and aminated residues (STYHCNGQ) in green and unusual amino/imino acids in grey (Larkin *et al.*, 2007).

# III. Discussion

We applied a tandem affinity purification strategy followed by a tandem mass spectrometry analysis to identify possible interacting proteins of Gtr1. The analysis of samples grown in the presence and in the absence of leucine allowed us to identify possible positive and negative (respectively) modulators of EGOC activity. Among the different candidates, the leucyl tRNA synthetase Cdc60 was the most striking interactor of Gtr1. Cdc60 directly binds leucine and, as a consequence, is a good candidate for a leucine sensor. Subsequently, Cdc60 was confirmed as a specific leucine-dependent interactor by co-immunoprecipitation experiments. Surprisingly, the catalytic charging activity of Cdc60 is not required for TORC1 activity and we could show that the editing (CP1) domain of Cdc60 is a major regulator of Gtr1 activity. In a conceivable model, under leucine rich conditions, the CP1 domain of the LeuRS might prevent inactivation of Gtr1 by a potential negative regulator, such as a GAP. Upon leucine depletion, the conformational change adopted by the CP1 domain, which results from mischarging events, disrupts the interaction between Cdc60 and Gtr1, allowing access of a putative GAP to Gtr1 (Figure 35).

According to our data, we cannot exclude that there are further levels of regulation of TORC1 by leucine, possibly through extracellular sensing, and/or through the regulation of Gtr1 GAP and GEF activities. Thus, it would also be appealing to assess Vam6 GEF activity from leucine starved and replete cells. It has been shown recently that, in *Schizosaccharomyces pombe*, the function of Vam6 in TORC1 signalling is conserved, and additionally, Vam6 interacts directly with Gtr1 in a leucine-dependent manner indicating that Vam6 binding to EGOC is indeed regulated (Valbuena *et al.*, 2012). Thus, it is tempting to imagine that Cdc60, instead of preventing GAP access, recruits Vam6 to the vicinity of EGOC to act on Gtr1. This question could be investigated through two hybrid interaction and co-immunoprecipitation experiments. Interestingly, over-expression of the Cdc60 CP1 domain in a *vam6Δ* mutant does not improve growth on rapamycin plates (data not shown).

The search of a Cdc60 mutant constitutively interacting with Gtr1 should also give insights into the regulation of TORC1 in response to leucine depletion. Such a mutant might become unresponsive to leucine deprivation and could also serve to study possible genetic interactions between Cdc60 and other proteins that could play a role in the TORC1 signalling pathway.



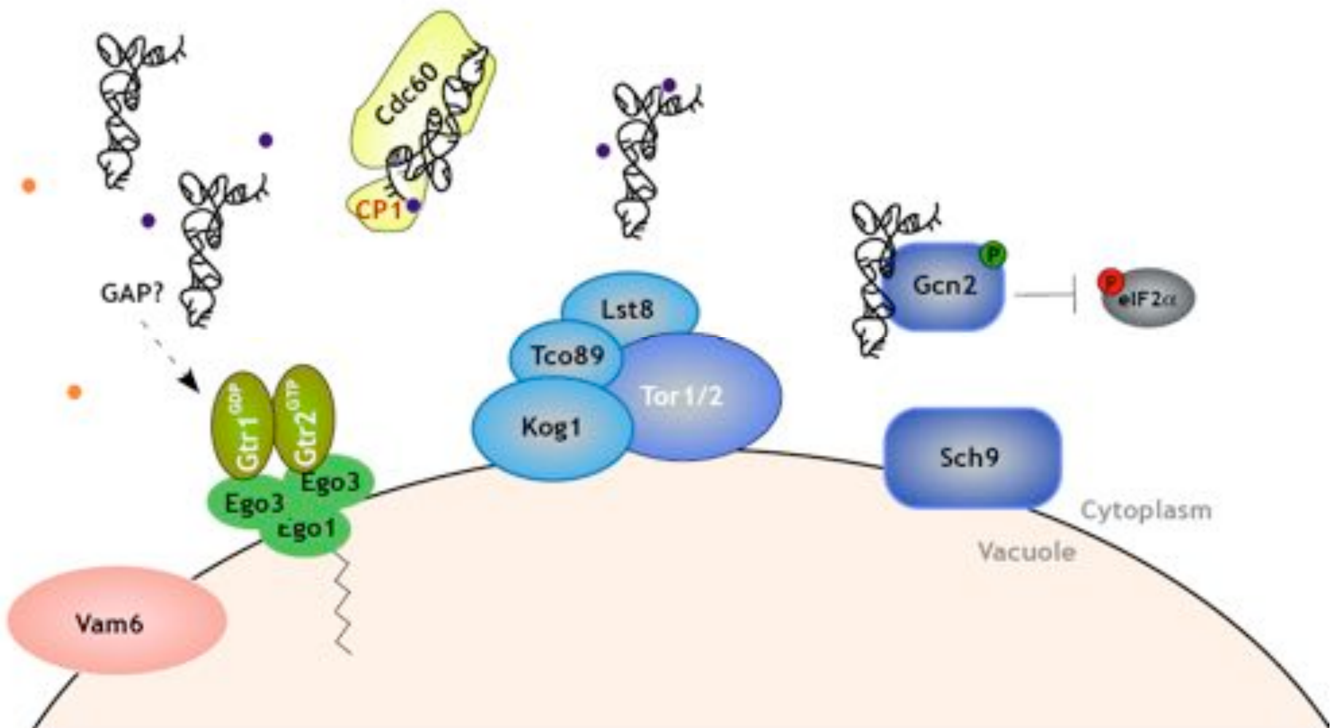




**Figure 35: model for TORC1 regulation by leucine**
Under leucine-rich conditions, Cdc60 editing activity is off and Cdc60 can interact with Gtr1 through its editing domain (CP1) (upper panel). Upon leucine depletion, Cdc60 mischarges $tRNA^{leu}$, which induces a conformational change and activation of the CP1 domain, disruption of Cdc60/Gtr1 interaction, inactivation of Gtr1 and consequently of TORC1 signalling (lower panel).

The implication of LeuRS in a function that differs from protein synthesis confirms previous observations showing that the function of this class of enzyme is not solely restricted to translation. Notably, the human tyrosyl-tRNA synthetase (TyrRS) contains an endothelial monocyte–activating polypeptide II (EMAP II) domain at its C-terminus. Thus, upon apoptosis, the protein is cleaved in two fragments with distinct cytokine activities (Wakasugi and Schimmel, 1999). Furthermore, the lysyl-tRNA synthetase (KRS) displays a role in the immune system (Lee *et al.*, 2004; Park *et al.*, 2005b; Yannay-Cohen *et al.*, 2009). The glutamylpolyl-tRNA synthetase is phosphorylated upon IFNγ release and consequently silences translation of specific mRNAs (Sampath *et al.*, 2004). The glutaminyl-tRNA synthetase mediates anti-apoptotic activity (Kim S., J biol Chem 2001). Finally, the methionyl-tRNA synthetase coordinates rRNA synthesis (Ko *et al.*, 2000). Additionally, the scaffold proteins AIMP1/43, AIMP2/p38 and AIMP3/p18 of the multi-tRNA synthetases complex are implicated in TGFβ signalling, ubiquitin delivery and tumor suppression, and p53 activation upon DNA damage, respectively (Choi *et al.*, 2009; Kim *et al.*, 2003; Lee *et al.*, 2008; Park *et al.*, 2005a).

While our report came into press, another research article that also proposed a role for the LeuRS in TORC1 signalling was published. This article from the laboratory of Kim Sunghoon proposed that LARS1, the human homologue of Cdc60, was a GAP for RagD, one of the two Gtr2 human homologues. From the observation that a fraction of Lars1 is associated with the lysosomes, they wondered about the role of LARS1 in TORC1 signalling (Han *et al.*, 2012). Based on co-localisation studies, immunoprecipitation and silencing experiments, they showed that the leucyl tRNA synthetase, specifically, acts as a leucine sensor on lysosomes to control TORC1. They found that LARS1 interacts specifically with mTOR and Raptor in a leucine-dependent manner. Knockdown of LARS1 decreases mTORC1 activation following amino acid and leucine stimulation and impairs mTOR and Raptor lysosomal relocalisation. Furthermore, LARS1 associates with RagD, preferentially in its GTP-bound state or following leucine stimulation, and overexpression of LARS1 stimulates the interaction between RagD and Raptor, while LARS1 silencing decreases such an interaction. Mutations in LARS1, which prevent leucine accommodation within the leucine-binding pocket, disrupts the interaction with RagD. In addition, the C-terminal fragment of LARS1 interacts with GTP-bound, but not GDP-bound, RagD, and LARS1 displays GAP activity towards RagD. Finally, mutations within the LARS1 leucine-binding pocket prevent GTP hydrolysis by RagD and subsequent mTORC1 activation following leucine stimulation.

This study shows that the mechanism of leucine sensing is conserved throughout evolution, and places the LeuRS as a key player in TORC1 signalling pathway. It also brings a rationale for the importance of leucine in growth control, without excluding the existence of possible additional amino acid sensing mechanisms. Although the results of this study differ slightly from our observations, we cannot rule out that Cdc60, while binding Gtr1 and preventing GTP hydrolysis in Gtr1, promotes

GTP hydrolysis in Gtr2 at the same time. Similarly, it is possible that LARS1 exerts its GAP activity towards RagD while preventing RagB inactivation.

Moreover, the phenylalanine 50 and tyrosine 52 in LARS1 are conserved in all eukaryotes, as is the serine 414 in Cdc60 (Figure 36). In spite of the similarities between the two reports, few observations indicate that the mechanisms of regulation between mammalian and yeast Rag GTPases would differ. Notably, Han *et al.* determined that LARS1 interacts with RagD via a necessary and sufficient C-terminal fragment spanning amino acids 784 to 1008. This observation is odd given the fact that, according to the general structure of LeuRS, this C-terminal fragment corresponds to the anticodon-binding domain, a region that makes extensive contact with the $tRNA^{leu}$. Still, we can imagine that the lysosomal fraction of LARS1 does not bind anymore $tRNA^{leu}$, potentially because of its engagement in GAP activity. The catalytic arginine 845 can only be found in mammals speaking against a role of Cdc60 as a Gtr2 GAP in *S. cerevisiae* (Figure 36).

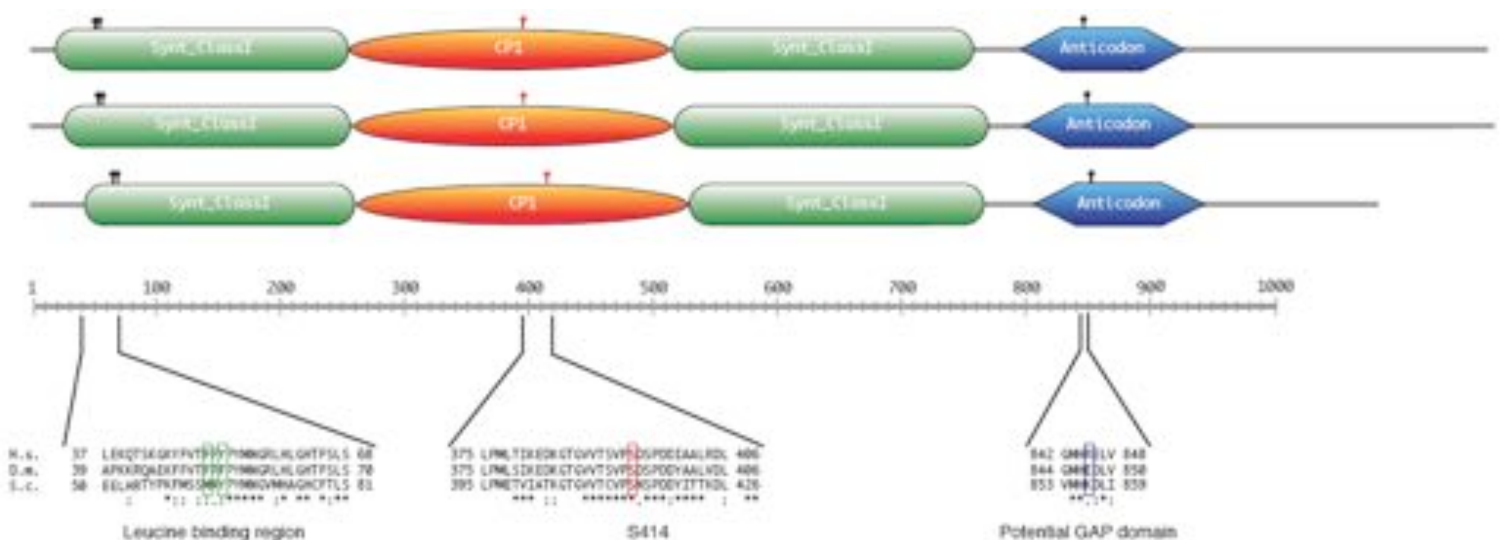



**Figure 36: Comparison of the organization and conservation of LeuRS from different organisms**
Schematic representation of the domain architecture of different LeuRS (H.s.: *Homo sapiens*; D.m.: *Drosophila melanogaster*; S.c. *Saccharomyces cerevisiae*) with the ClustalW2 alignment of the regions of LeuRS proposed to be implicated in the regulation of Rag GTPases.

In addition, the conservation of the ArfGAP motif is doubtful as seen in the alignment of some ArfGAPs domains, chosen by *Han et al.*, with LARS1 (Figure 37). We can see that with the exception of few residues, among which the putative catalytic arginine 845, there is poor homology between LARS1 and the different ArfGAP, while those GAPs have a high similarity.

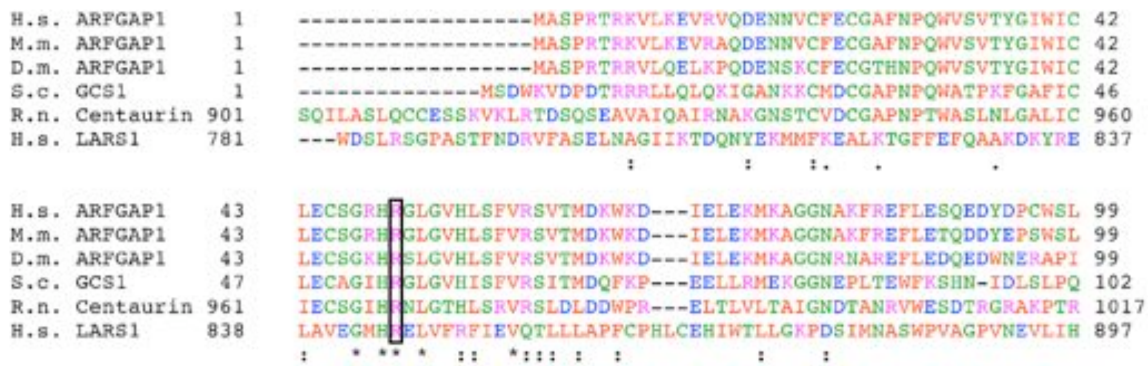



**Figure 37: sequence alignment of Lars1 putative GAP domain with known Arf-GAP domains**
ClustalW2 alignment of Lars1 putative GAP domain with Arf-GAP domains from different organisms and surrounding regions. The putative catalytic arginine 845 is black-boxed (H.s.: *Homo sapiens*; M.m. *Mus musculus*; R.n. *Rattus norvegicus*; D.m.: *Drosophila melanogaster*; S.c. *Saccharomyces cerevisiae*).

This suggests that the function of LARS1 as a RagD GAP arose latter in evolution, and that this mechanism of regulation of the mTORC1 signalling pathway is specific to mammals. As a consequence, this also raises the question of the means by which other organisms regulate Gtr2/RagD loading status.

In addition to the leucyl-tRNA synthetase, we have shown that the two other branched-chain amino acyl-tRNA synthetases CP1 domains also interact with Gtr1 by two-hybrid. Although the role of these two enzymes has not been investigated further yet, it would be very interesting to expand the mechanism described for Cdc60. Importantly, branched chain amino acids are known to favour protein synthesis (Buse *et al.*, 1979; May and Buse, 1989; Patti *et al.*, 1998; Xu *et al.*, 1998). Han *et al.* observed that LARS1, apart from leucine, is able to bind, to a lesser extent, isoleucine and valine, indicating that LeuRS might be a general sensor of BCAA, but this point will need clarification. In addition, it is possible that other BCAARS act as GAPs for RagC.

Furthermore, the class I methionyl-tRNA synthetase and some class II tRNA synthetase also display an editing activity (*e.g.* threonyl-, alanyl-, prolyl-, phenylalanyl-, lysyl- and seryl-tRNA synthetases). While the structure of these enzymes indicates that the editing domain is not clearly separated from the charging domain, as is the case for the BCAARS, they might still function as TORC1 regulators.

Finally, investigating the role of each amino acid in TORC1 activation would be interesting and yeast should be a powerful system to study such a question, as it would simply require deletion of one or a few genes in each amino acid biosynthetic pathway to render cell auxotroph for one amino acid. In mammals, it has been observed that leucine alone cannot account for the whole activation of mTORC1, and that additional amino acids must play a role (Fox *et al.*, 1998; Shigemitsu *et al.*, 1999a).

For example, arginine is a strong activator of TORC1 in some cell types, and it was shown to induce S6K phosphorylation and to enhance cell migration in enterocytes (Rhoads *et al.*, 2007). It also stimulates protein synthesis in skeletal muscles (Yao *et al.*, 2008a) and is required for uterine

implantation of the embryo through TORC1 activation (Gonzalez *et al.*, 2012; Zeng *et al.*, 2008). In yeast, arginine is a very abundant amino acid, mainly present in the vacuole. However, it is described as a poor nitrogen source (Godard *et al.*, 2007).

Glutamine is also required for TORC1 activation and more specifically glutamine uptake is necessary for the import of leucine and consequent activation of mTORC1 (Nicklin *et al.*, 2009). However, it is not known whether intracellular glutamine can also directly activate TORC1. As seen in chapter III, glutamine definitely plays a major role in the stimulation of TORC1 in yeast, but the precise mechanism remains to be elucidated. Further studies for the role of other tRNA synthetases in the control of TORC1 and cell growth will be required. It will also be necessary to determine the precise role of each amino acid for stimulation of the pathway and the molecular mechanisms that underlie activation. Additionally, it will be important to discriminate between the role of extra and intra-cellular pools of amino acids. Finally, uncovering how the loading status of Gtr1 and Gtr2 is regulated and if these regulators are themselves regulated will increase our understanding of the TORC1 signalling pathway. Ultimately, identifying the homologues of Gtr1/2 regulators in mammals will strongly reinforce our global understanding of cell growth control and proliferation, and the molecular mechanisms that might lead to cancer development via mTORC1.

# - General Discussion -

# General discussion

## 1. On the role of glutamine

Several lines of evidence point toward glutamine as a key amino acid controlling TORC1 signalling. As mentioned previously, glutamate and glutamine link several metabolic pathways, which place them at the interface of nitrogen and carbon sources, and as such they may control cellular growth. We have demonstrated in chapter II that these amino acids, as previously proposed, play an important role in controlling TORC1 activity, and depletion of glutamine leads very rapidly to an inactivation of TORC1. However, glutamine activation of TORC1 is independent of the EGOC and the means by which it stimulates TORC1 activity remains unclear.

Glutamine controls yeast cell growth through at least three different signalling pathways that are potentially overlapping, and which involve the NCR pathway, the GAAC and the RTG pathway. Notably, the TORC1-dependency of Gln3 is unclear, and a mapping of Gln3 phospho-residues and a better understanding of the kinases acting on Gln3 should improve our understanding of NCR gene regulation by Gln3. Moreover, activation of Gln3 and Gat1 seems to depend on different signals. An attractive model proposes that Gln3 would be activated under a mild stringent stress, and Gat1 activation might arise following a harsher stress (Zaman *et al.*, 2008). Recently, a large-scale study of the protein kinase and phosphatase network in yeast allowed the identification of the protein Nnk1 (Nitrogen Network Kinase) as a kinase for Ure2. Nnk1 activity antagonizes Ure2-Gln3 interaction. Moreover, Nnk1 over-expression renders cells hypersensitive to rapamycin on plates, and constitutively targets Gln3 to the nucleus. Lastly, Nnk1 associates with the TORC1 subunits Tco89 and Kog1, and with the $NAD^+$-dependent glutamate dehydrogenase Gdh2 that converts glutamate to $\alpha$-ketoglutarate and ammonia, and Gdh2 is a substrate for Nnk1 *in vitro* (Breitkreutz *et al.*, 2010). These observations place Nnk1 as a very interesting candidate and, possibly, a missing link in the control of NCR gene expression in response to amino acid, and more particularly, glutamine depletion (Figure 39).

As for Gln3, the relationship between TORC1 and the RTG pathway is ambiguous. The link between glutamate/glutamine, TORC1 and RTG is unclear, and further investigations are required to elucidate whether glutamate/glutamine act in parallel on the two pathways, and if connections exist. Interestingly, it has been shown recently that Mks1 directly interacts with the kinase Fmp48, but no direct phosphorylation event could be shown (Breitkreutz *et al.*, 2010). Notably, Fmp48 can be purified from yeast mitochondrial fractions (Reinders *et al.*, 2006). Cells over-expressing Fmp48 have a growth defect on nonfermentable carbon sources, abnormal mitochondrial morphology and repress genes encoding TCA cycle enzymes, subunits of the ATP synthase and components of the electron transport chain. Interestingly, Fmp48 directly interacts with Tor1 and Tor2 kinases, and over-expression of Fmp48 confers rapamycin resistance on plates. Finally, the kinase activity of Fmp48 is

increased upon rapamycin treatment (Breitkreutz *et al.*, 2010). All these data suggest that Fmp48 might be an intermediate between mitochondria, TORC1 and the RTG pathways and that it may be activated by mitochondrial dysfunction (Figure 38).

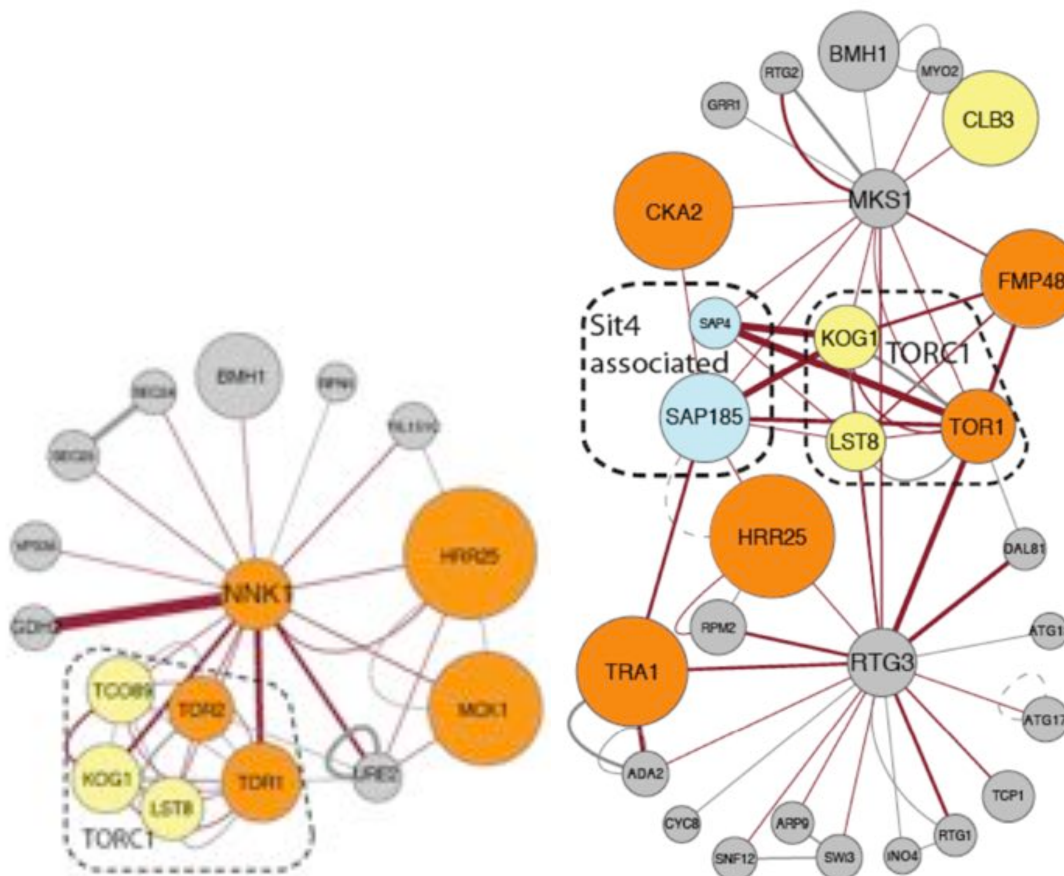



**Figure 38: Network of Nnk1 and Fmp48 kinases associated proteins (Breitkreutz *et al.*, 2010)**
Kinases are in orange, phosphatases in blue, kinase-associated proteins in yellow, and other proteins in gray. Kinase-phosphatase interactions are connected by a red line, low-throughput interactions by a gray line and high-throughput high confidence interactions by a gray dashed line. Dashed circled proteins indicates known interacting proteins. The size of a node is proportional to the number of interactions in the dataset and the size of the lines indicates the peptide count of interaction.
(A) The kinase Nnk1 interacts directly with TORC1 and NCR components.
(B) The kinase Fmp48 interacts directly with TORC1 and RTG components, thus providing a possible link between the two pathways.

## 2. On the role of leucine

Several studies place leucine as a very strong TORC1 activator, but the precise mechanisms of stimulation have remained elusive for a long time (Anthony *et al.*, 2000; Buse and Reid, 1975; Kimball *et al.*, 1999; Lynch *et al.*, 2000; Patti *et al.*, 1998; Shigemitsu *et al.*, 1999b; Xu *et al.*, 1998). Recently, several reports shed light on the yeast EGOC, and its mammalian counterpart, the Rag-Ragulator complex, as a key player relaying the amino acid signal to TORC1 (Binda *et al.*, 2009; Kim *et al.*, 2008; Sancak *et al.*, 2010; Sancak *et al.*, 2008). Upon amino acid stimulation, the small GTPase Gtr1 preferentially in its GTP bound state interacts at the vacuolar rim with the TORC1 subunit Kog1 to stimulate TORC1 activity (Binda *et al.*, 2009). In this process, it has been proposed that leucine is

the most potent activator of TORC1. In chapter III, our results demonstrated that the leucyl-tRNA synthetase Cdc60 directly interacts with Gtr1 in a leucine-dependent manner. Furthermore, we have shown that the interaction occurs via the editing domain of Cdc60, whose conformation dictates its interaction with Gtr1. Whether leucine binding in Cdc60 is a requisite for the interaction between Cdc60 and Gtr1 is not clear, although our leucinol experiment tend to show that occupation of the leucine binding domain within Cdc60 is sufficient to mediate a signal.

To understand the reason why cells respond much more strongly to leucine that any other amino acid, it is important to note that leucine is the most abundant amino acid in proteins while its intracellular levels remain relatively low as compared to other amino acids (Echols N. *et al.*, 2002; Kitamoto *et al.*, 1988). Thus, monitoring the level of the potentially limiting amino acid (i.e. leucine) would represent an economic way for the cell to estimate the global level of all amino acids. In accordance with such an assertion, the leucyl-tRNA synthetase is the most abundant amino acyl-tRNA synthetase in yeast cells, and as such would potentially be the best sensor.

However, yeast cells still respond, although to a lesser extent, to a limitation of other amino acids, such as histidine or lysine, in a Gtr1-dependent manner. Moreover, as we have seen, yeast cells respond very strongly to glutamine depletion apparently independently of the EGOC, indicating that there are additional amino acids sensing mechanisms that impinge on TORC1. Further investigations are thus required to fully decipher the molecular mechanisms behind amino acid control of TORC1.

### 3. Regulation of the intracellular amino acid levels

Intracellular amino acids control many cellular responses to allow yeast cells to adapt to nitrogen availability and quality. Amino acid transporters influence TORC1 activity by modulating the intracellular content of amino acids. In addition, the composition of amino acid transporters at the plasma membrane is modulated by the SPS transceptor system, which controls the expression of amino acid permeases. In turn, TORC1 monitors the stability of amino acid permeases at the plasma membrane to ensure sufficient and continuous amino acid uptake required for optimal growth.

In mammals, the expression of amino acid transporters is regulated by growth factors at the translational and transcriptional levels (Edinger *et al.*, 2003; Edinger and Thompson, 2002). In contrast, how the trafficking and degradation of these proteins is regulated remains unknown. Nevertheless, amino acid uptake is required to stimulate mTORC1. Notably, the bidirectional system L transporter is required for cellular entry of BCAA such as leucine (Verrey *et al.*, 2004), and is highly expressed in many tumors (Storey *et al.*, 2005). The system A also plays an important role by coupling intracellular amino acid accumulation with $Na^{+}$ export. The means by which amino acid transporters control mTORC1 activity has been partly elucidated with the finding that glutamine efflux is a requisite for extracellular leucine uptake (Nicklin *et al.*, 2009).

## 4. Regulation of the EGOC?

As discussed in chapter III, the leucyl-tRNA synthetase (LeuRS) is also a leucine sensor and a modulator of TORC1 activity in mammalian cells, although the mechanism seems to be different. Indeed, Han *et al.* describe a mechanism where the LeuRS act on RagD as a GAP. In yeast, the role of Gtr2 inside the EGOC is unclear. We know from previous experiments that Gtr1 stimulates TORC1 in response to amino acids. However, under such conditions Gtr2 would rather be inactive. When bound to GTP (i.e. upon amino acid depletion), Gtr2 is inhibitory to TORC1. We can speculate that following amino acid deprivation, Gtr2 activation actively participates in TORC1 inactivation. Another possibility could be that upon activation, Gtr2 stimulates processes under TORC1 repression such as autophagy or activation of phosphatases. To further elucidate the role of Gtr2, it would be interesting to find some specific interactors. A previous two-hybrid screen identified the proteins Fpr3 and Ptp1 as potential Gtr2 interactors (Dubouloz, PhD thesis 2006). Fpr3 is a peptidyl-prolyl cis-trans isomerase and Ptp1 is a phosphotyrosine-specific protein phosphatase that dephosphorylates Fpr3 among many proteins (Wilson *et al.*, 1995). In addition, Ptp1 was recently shown to be an inhibitor of filamentous growth. Pseudohyphal growth usually occurs when a fermentable carbon source is limiting. Thus, it would be interesting to test whether amino acid limitation might also trigger filamentous growth and if Gtr2 might be required to promote such growth.

In addition, deciphering the GAP and GEF regulators of Gtr1 and Gtr2 may give invaluable informations for our understanding of amino acid regulation of the TORC1 pathway. We already know that Vam6 acts as GEF for Gtr1. However, the Gtr1 GAP remains unknown. Early evidence in our laboratory tends to indicate that the protein Iml1, in a complex with Npr2 and Npr3, acts as a GAP for Gtr1 at the vacuolar rim. Notably, Iml1 deletion induces hyperactivation of TORC1, while Iml1 over-expression downregulates TORC1 activity. Moreover, Iml1 displays, *in vitro*, a GAP activity towards Gtr1 (Panchaud N., personal communication). Nevertheless, regulators of Gtr2 remain to be discovered. In addition, how amino acids may regulate the activity of these proteins remains to be addressed.

Another point of interest that remains to be elucidated concerns the means by which EGOC stimulates TORC1. In mammals, it is thought that the Ragulator complex recruits the Rag GTPases to lysosomes. Amino acids stimulate GTP loading on RagA/B which can bind Raptor and, hence, recruits mTORC1 to lysosomes where it can be activated by Rheb (Kim and Guan, 2011). In yeast, it seems unlikely that the Rheb homologue, Rhb1, performs the same function. Moreover, yeast TORC1 always localizes to the vacuolar rim indicating that the mechanism of regulation may differ from higher eukaryotes. As discussed previously, the activity of TORC1 would depend on the loading status of the Gtr proteins, which are regulated by GAPs and GEFs.

## 5. Emerging roles for amino acyl-tRNA synthetases (AARSs)

### *i AARSs as signalling molecules*

In higher eukaryotes, some AARSs form a multiprotein complex composed of 9 synthetases (Isoleucyl-, leucyl-, lysyl-, methionyl-, asparagyl-, arginyl-, prolyl-glutamyl- and glutaminyl-tRNA synthetases) and 3 scaffold proteins (AIMP1, 2 and 3)(Lee, 2004; Rho *et al.*, 1999). The apparent grouping of these enzymes in a single complex might represent a mean to improve the efficiency of the translation process (Kyriacou and Deutscher, 2008), but it may also represent a pool of regulatory factors that play a role in signalling apart from their synthetase function (Ray *et al.*, 2007). For instance, the glutaminyl-prolyl-RS fusion protein is released from the complex upon IFN-γ stimulation to repress translation of mRNAs implicated in the inflammatory response (Sampath *et al.*, 2004). The free Methionyl-RS promotes RNA synthesis in the nucleolus (Ko *et al.*, 2000). The glutamyl-RS regulates the apoptosis response (Ko *et al.*, 2001). The lysyl-RS is secreted to induce the pro-inflammatory response (Lee *et al.*, 2004; Park *et al.*, 2005b; Yannay-Cohen *et al.*). Moreover, secreted AIMP1 participates in angiogenesis (Lee *et al.*, 2006b; Park *et al.*, 2002), inflammation (Ko *et al.*, 2001; Park *et al.*, 2002), glucose metabolism (Park *et al.*, 2006b) and wound healing (Han *et al.*, 2006; Park *et al.*, 2005c). The AIMP2 protein plays a role in the control of cell death and cell differentiation (Han *et al.*, 2008; Kim *et al.*, 2003), and AIMP3 participates in the DNA damage response and genomic stability via ATM/ATR (Park *et al.*, 2005a; Park *et al.*, 2006a). In addition, although they are not part of the complex, the tryptophanyl- and tyrosyl-RS act as procytokines (Wakasugi and Schimmel, 1999; Yang *et al.*, 2004).

In yeast, our study is the first demonstration of a signalling role for an AARS that differs from its aminoacylation function. We may speculate that, although it has not been studied yet in yeast, other AARSs may have such divergent function. In *S. cerevisiae*, the only AARSs known to associate in a complex are the glutamyl- and prolyl-RS Gus1 and Mes1, respectively. These enzymes apparently do not interact directly, but are anchored together in the cytoplasm by the protein Arc1. Recent findings tend to show that upon diauxic shift, or following a shift from a fermentable to a non-fermentable carbon source, Gus1 and Mes1 are released from Arc1. Targeting of Gus1 to the mitochondria allows production of Gln-$tRNA^{Gln}$ required for the transcription of mRNAs of enzymes involved in the respiratory chain. On the other hand, free Mes1 enters the nucleus and triggers expression of genes also involved in the mitochondrial respiration (Becker H., personal communication).

*ii AARS and their connections to diseases*

Considering the various natures of their alternative functions, it is not surprising that AARSs are associated with a wide variety of human diseases from neurological disorders to autoimmune diseases and cancer. This includes Charcot-Marie-Tooth disease provoked by mutations in the glycyl- and tyrosyl-RS (Antonellis *et al.*, 2003; Jordanova *et al.*, 2006). Certain forms of ataxia are linked to editing-defective alanyl-RS (Lee *et al.*, 2006a; Williams and Martinis, 2006), while certain leukoencephalopathies are caused by mutations in the aspartyl-RS (Scheper *et al.*, 2007). Furthermore, the “antisynthetase syndrome” is caused by the production of autoimmune antibodies, notably against histidyl-, alanyl-, threonyl-, phenylalanyl- glycyl and asparagyl-RS (Hirakata *et al.*, 1999; Mathews and Bernstein, 1983; Mathews *et al.*, 1984).

In addition, abnormal expression of AARSs has often been connected to tumor development and cancer. Thus, mutations in the promoter region of isoleucyl-RS modify its expression pattern and were found to correlate with hereditary non-polyposis colorectal cancer and Turcot syndrome (Miyaki *et al.*, 2001). Preferential expression of the $\alpha$ subunit of phenylalanyl-RS is associated with lung solid tumors and leukemia (Lieber *et al.*, 1976; Rodova *et al.*, 1999). The lysyl-RS is overexpressed in breast cancer (Park *et al.*, 2005b), and the glycyl-RS in papillary thyroid carcinoma (Scandurro *et al.*, 2001; Wasenius *et al.*, 2003).

More recently, while screening for differentially expressed genes in lung cancer cell lines, Shin *et al.* found that the leucyl-RS (Lars1) is overexpressed in 59% of primary lung cancer tissues. Lung cancer cells in culture treated with siRNAs against *LARS1* reduces the formation of colonies as well as their size and migration capacity (Shin *et al.*, 2008). This study together with the finding that Cdc60/Lars1 control Gtrs/Rags-TORC1 in response to leucine raises the possibility that TORC1 is hyperactivated in primary lung cancers, and places Lars1 as a potential therapeutic target.

# - Materials and Methods -

**Strains, Growth Conditions and Plasmids**

Unless stated otherwise, yeast and bacteria media were prepared according to standard recipes (Rose *et al.*, 1990; Sambrook, 2001). Yeast strains were grown at 30°C either in full YPD medium (1% yeast extract, 2% bacto-peptone and 2% w/v glucose) or in selective synthetic medium without amino acids (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulphate, and 2% glucose) or in selective medium (0.67% yeast nitrogen base without amino acids, and 2% glucose) supplemented with the appropriate requirement. Solid medium contained additionally 2% w/v agar. For selection of kanamycin resistant strain, geneticin (Calbiochem) was added to a final concentration of 100 μg/mL.

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 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.

Bacterial cultures were grown at 37°C on Luria Bertani medium (LB; 1% w/v NaCl, 1% Bacto-tryptone, 0.5% w/v yeast extract). Solid medium contained additionally 2% w/v agar. For ampicillin selection, ampicillin (Applichem) was added to a final concentration of 100 μg/mL. Plasmid manipulations were performed in *Escherichia Coli* strain DH5α using standard procedures (Sambrook, 2001). Standard procedures of yeast genetics and molecular biology were used (Guthrie and Fink, 2002; Rose *et al.*, 1990; Sambrook, 2001)

**Chemicals:**

Rapamycin was purchased from LC laboratories and used at a final concentration of 200ng/mL unless stated in the text. Cycloheximide was purchased from Fluka and used at a final concentration of 25 μg/mL. N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), 2-Nitro-5-thiocyanato-benzoic acid (NTCB), norvaline, leucinol, mycophenolic acid and 1,3-dihydro-1-hydroxy-2,1-benzoxaborole (DHBB) were purchased from Sigma-Aldrich.

**Extraction of the vacuolar amino-acids pools**

The protocol is adapted from Oshumi (Ohsumi *et al.*, 1988) with minor adaptations. Cells (~ 10 $OD_{600}$) were harvested by filtration and washed 3 times with distilled water. They were then resuspended and incubated during 10 min at room temperature in 3 mL of extraction buffer (2,5mM Potassium phosphate; 0,6M sorbitol; 10mM glucose ; 0.2mM $CuCl_2$ ; pH 6,0). After permeabilization, cells were washed 3 times with 15 mL of wash buffer (2,5mM Potassium phosphate; 200mM KCl; 10mM $CaCl_2$; 5mM $MgCl_2$, pH 6,0), resuspended in 1mL of water and boiled for 15 min. The suspension was centrifuged for 5 min at 13,000 rpm and the supernatant was filtered through 0.22 μm filters and kept at -20°C for subsequent injection.

**Whole-cell amino- acids analysis**

Cells (~ 10 $OD_{600}$ ) were harvested by filtration and washed 3 times with distilled water. They were suspended in distilled water, boiled for 15 min and finally centrifuged 10min at 13,000 rpm. The supernatant was finally filtered through 0.22 µm filters and kept at -20°C for subsequent injection.

**Chromatographic separation and quantification of amino acids**

Vacuolar and whole-cell amino-acids pools were quantified with pulsed electrochemical detection after separation by anion exchange chromatography (Column Dionex Aminopac PA10 2X250 mm) with a AAA-direct, Dionex Amino Acid Analyser, using a sodium acetate gradient to increase the ionic strength.

**Sch9 phosphorylation analyses**

To analyze Sch9T570A-HA5 C-terminal phosphorylation, we used the chemical fragmentation analysis as described previously (Urban *et al.*, 2007; Wanke *et al.*, 2008). Briefly, 10 $OD_{600}$ of cells' 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, 1mM PMSF, and 0.5x PPi) with glass beads in a bead beater (3 X 30 sec, 5000 rpm, 60 sec pause) with subsequent heating for 10 min at 65°C. For NTCB cleavage 30 µl of 0.5 M CHES (pH 10.5) and 20 µl of NTCB (7.5 mM in H2O) were added and samples incubated overnight at RT before 1 volume of 2X sample buffer (+ 0.1 M DTT) was added. Further analysis was done by 7.5% SDS-PAGE and immunoblotting using anti-HA antibody 12CA5.

**Determination of the chronological lifespan:**

Yeast cells were grown at 30°C without replacing the growth medium. Aliquots were regularly taken, serially diluted and plated on YPD agar rich medium. After three days of incubation at 30°C, the colony forming units per mL of culture (CFU/mL) were scored (number of colony X dilution factor) and expressed as a percentage of the initial value. The results are plotted on a logarithmic scale.

**eIF2α Phosphorylation Analyses**

For the analysis of the phosphorylation status of eIF2α, 10 $OD_{600}$ of cells' 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, 1mM PMSF, and 0.5x $PP_i$) with glass beads in a bead beater (3 X 30 sec, 5000 rpm, 60 sec pause) with subsequent heating for 10 min at 65°C. Equal amounts of total protein 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 re-probed with polyclonal anti-eIF2α antibodies.

**Tandem Affinity Purification (TAP)**

Gtr1-TAP was purified, using a standard TAP-tag purification protocol (Gelperin *et al.*, 2005), from wild-type (YL515) cells harboring plasmid pMB1344-*GTR1-TAP*. Around 4000 $OD_{600}$ of cells were harvested by centrifugation 5 min. at 5000 g and washed once with 50 ml cold water and once with 25 ml cold TAP buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5mM $MgCl_2$, 1mM DTT) containing 0,5 mM PMSF and 1 x protease inhibitor (Roche). Cells were centrifuged 10 min at 3000 g and frozen in liquid nitrogen.

The pellet was carefully thawed on ice and resuspended in TAP buffer containing 0,5 mM PMSF and 1 x protease inhibitor (Roche). The suspension was mixed with the same amount of glass beads (diameter 0,4-0,6 mm) and lysis was performed in a Pulverisette at 500 rpm, 4 min twice with 1 min break. The supernatant was spinned at 3000 g for 10 min, transferred to ultracentrifugation tubes and spinned for 1h at 100.000g. The fatty top phase was removed and lysate transferred to a new tube with pre-washed IgG-coated sepharose beads (in TAP buffer). After 1 h of incubation on a rotating wheel, beads were washed with 15 mL of TAP buffer. Beads were then resuspended in 150 μl of TAP buffer containing 0.5 mM DTT and 5 μl of TEV (1 mg/ml) for elution, overnight at 4°C on a turning wheel.

A second step of purification was performed using calmodulin beads. Basically, $CaCl_2$ was added to the previous TEV eluate at a final concentration of 2 mM and the eluate was incubated for 1h at 4°C with 0.5 mL slurry of calmodulin beads (Stratagene) pre-equilibrated in calmodulin buffer (TAP buffer containing 1 mM DTT and 2 mM CaCl2). Beads were washed with 15 mL of calmodulin buffer and proteins were eluted in TAP buffer containing 5 mM EGTA at 30°C for 20 minutes with shaking. An additional 10 min step of elution was performed and eluates were combined.

**Mass spectrometry analysis**

Control and TAP preparations were resolved on a 10% SDS-PAGE and stained with coomassie blue. The gel was cut in six molecular weight regions of equal size and after extraction and trypsin digestion of the bands, each fraction was analysed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). The colleciton of spectra are submitted to database search and identified proteins are submitted to statistical validation.

**Co-immunoprecipitation experiments**

For Cdc60-$HA_3$ coIP experiments with Gtr1, Gtr1-TAP and Igo1-TAP were purified from 200 $OD_{600}$ cells filtered on a 0.2 μM membrane. Lysates were incubated for 1h at 4°C with IgG-coated sepharose beads that were previously washed with lysis buffer (20 mM HEPES pH=7.5, 110 mM KoAc., 100 mM NaCl, 5mM MgCl2, 0.5% NP40) containing protease inhibitor cocktail (1x; Roche) and 0.5 mM PMSF.

For Kog1-$HA_3$ and $His_6$-$HA_3$-$Cdc60^{CP1}$ coIP experiments with Gtr1, the conditions are essentially the same except that the lysis buffer composition is the following: 20 mM HEPES pH=7.5, 300 mM NaCl, 5mM MgCl2, 0.5% NP40.

Beads were then washed 3 times with lysis buffer with an incubation period of 5 min on the wheel at 4°C prior to addition of loading buffer and subsequent heating at 65°C for 10 min.

**LexA yeast two-hybrid screen:**

The screen was performed essentially as described elsewhere (Cagney *et al.*, 2000). The open reading frame of *GTR1* was cloned into the pEG202-derived (bait) plasmid expressing LexA-Gtr1 fusion protein from the *ADH1* promoter. The bait plasmid was transformed into the EGY48 yeast strain containing the *LexAop-lacZ* reporter plasmid pSH18-34. The cDNA library of pJG4-5-derived (prey) plasmids expressing the VP16 AD fusion protein from the *GAL1* inducible promoter was introduced into the EGY48 pLexA-Gtr1 strain with a standard transformation procedure and plated on synthetic medium containing 1% raffinose 2% galactose without leucine. Remaining transformed cells were diluted $10^2$ to $10^4$ times and plated on SD without histidine and tryptophan An aliquot was plated on synthetic medium containing 1% raffinose 2% galactose with leucine to assess the total number of transformants.

**Split-ubiquitin yeast two-hybrid assay**

Proteins interactions were investigated using the membrane-based yeast two-hybrid system as described by the manufacturer (Dualsystems Biotech). Strain NMY51 containing the *LexAop-HIS3*, *LexAop-ADE2* and *LexAop-lacZ* reporter genes was co-transformed with the pCab (bait) plasmid expressing a CUB-LexA-DBD fusion protein expressed from the *CYC1* promoter together with a pPRN3-N (prey) plasmid expressing NUBG-HA fusion protein expressed from the *CYC1* promoter. Yeast cells were grown to mid-log phase in SD without leucine and tryptophan and assayed for β-galactosidase activity using a classical ONPG-based protocol (Stern *et al.*, 1984). Interactions were expressed as Miller-Units.

**Extraction, Separation and Analysis of Amino-Acylated tRNAs**

Assessment of $tRNA^{leu}$ charging was performed as described in (Köhrer and RajBhandary, 2008). Briefly, total RNA (from 10 $OD_{600}$ of cells) was extracted twice 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, immobilized by UV-crosslinking. Hybridisation was performed overnight at 42°C using $P^{32}$-labeled oligonucleotide probes that specifically bind $tRNA^{leu}$ (5'-CATCTTACGATACCTG-3') or 5S rRNA (5′-GTCACCCACTACACTACTCGG-3'). The corresponding membranes were then exposed at -80°C on X-ray films for autoradiography.

**Purification of GST-tagged protein from bacteria**

Expression of the tagged protein was induced by addition of 0.1 to 0.4 M of IPTG to cells grown at 37°C. After 3 hours of induction, cells were harvested by centrifugation, washed in 10 mL of PBS containing 1 X protease inhibitors and 1 mM of PMSF and sonicated 3 X 30 s with 2 min pauses on ice. Cell debris were pelleted and the supernatant was incubated on the wheel with pre-washed Glutathione sepharose resin. After three washing steps in PBS, GST fusion proteins were eluted by addition of 10 mM glutathione in PBS for 30 min at room temperature. Proteins were directly used for assay or kept frozen in 20% glycerol

***In vitro* kinase assay**

The TAP-tagged Tco89 was purified using IgG sepharose as described by Binda *et al.* (Binda *et al.*, 2009). Sch9-HA and its kinase dead version were purified from yeast. Cells were lysed as described ealier in lysis buffer (1 X PBS, 10% glycerol, 0.5% tween,) containing phosphatase inhibitors, protease inhibitor cocktail (1x; Roche) and 0.5 mM PMSF. Anti-HA antibodies (12CA5, Covance) were pre-incubated with protein G agarose and allow to bind for 30 min at 4°C on a rotating wheel. The cell lysate was incubated with the agarose beads for 1 h at 4°C and washed three times with 750 μL of lysis buffer at 4°C with 5 min incubation on the wheel in-between washes. The

purified proteins were directly used for activity test. Equal amounts of proteins were used for the enzymatic test. The proteins were resuspended in their respective lysis buffer containing 25 μM ATP and 10 mCi of g33P-ATP and kept on ice. The potential protein substrates were added and the reaction allowed to start by incubating the samples at 30°C. After 30 min the reaction was stopped by addition of 5 X loading buffer and boiled for 5 min. Samples were loaded on a SDS-PAGE, the gels were stained with coomassie blue, dried and exposed to X-ray cassette.

***Microscopic analyses***

Cells were imaged using an Olympus BX51 microscope (Olympus) equipped with a piezzo-positioner (Olympus), a XBO 75 W Xenon light source (Atlanta Light Bulbs Inc. GA), 100x 1.45 Plan-Fluar objectives, and a three-position filter sliding rack. Image acquisition was performed with a F-view2 camera (Olympus). The microscope and camera were controlled by CellM software (Olympus). The microscope was equipped with a complete set of filters: Filter set U-MWIBA, filter set U-MWIG, filter set U-MNUA2 (Olympus). To ensure that the filters were aligned, we utilized Tetraspeck Fluorescent Microspheres (Molecular Probes, Eugene, OR). We acquired 7-10 (0.5 μm apart) z-sections, which were projected to two-dimensional images and analyzed with CellM software. All live cell imaging were performed with mid-log phase cells cultured in SC complete media supplemented with appropriate nutrients for plasmids maintenance.

# - Strains and plasmids -

**Strains used in Chapter I**

| Strain name | Genotype | Source or Reference | Figure |
|---|---|---|---|
| BY4742 | *MATα*; *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* | Euroscarf | |
| YL515 | [BY4741/2] *MATα*; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | |
| BY4741 | *MAT***a**; *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* | Euroscarf | 16 A-C |
| YL516 | [BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | 9A-D; 10A-B; 11A-B; 12A-B; 13A-B; 14A-B; 15 |
| GB69 | [BY4741] *MAT***a** *atg22Δ::kanMX4* | Euroscarf | 12A-B |
| CDV1405 | [YL516] *MAT***a** *avt3Δ::kanMX4 avt4Δ::kanMX4* | | 12A-B |
| CDV1415 | [YL515] *MATα avt3Δ::kanMX4 avt4Δ::kanMX4 avt6Δ::kanMX4* | | 12A-B |
| GB1773 | [YL516] *MAT***a** *avt3Δ::kanMX4 avt4Δ::kanMX4 avt6Δ::kanMX4 atg22Δ::kanMX4* | | 11A-B; 12A-B; 13; 14A-B; 15 |
| MB1537 | [YL516] *MAT***a** *gtr1Δ::kanMX4* | (Binda *et al.*, 2009) | 14A-B |
| GB11 | [YL516] *MAT***a** *avt3Δ::kanMX4 avt4Δ::kanMX4 avt6Δ::kanMX4 atg22Δ::kanMX4 gtr1Δ::kanMX4* | | 14A-B |
| MJA2753 | [BY4741] *MAT***a** *vma1Δ::kanMX4* | Euroscarf | 16C |
| MJA2754 | [BY4741] *MAT***a** *vma2Δ::kanMX4* | Euroscarf | 16C |
| MJA2755 | [BY4741] *MAT***a** *vma3Δ::kanMX4* | Euroscarf | 16B-C |
| MJA2756 | [BY4741] *MAT***a** *vma8Δ::kanMX4* | Euroscarf | 16C |

**Strains used in Chapter II**

| Strain | Genotype | Source or Reference | Figure |
|---|---|---|---|
| BY4741 | *MAT***a**; *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* | Euroscarf | |
| BY4742 | *MATα*; *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* | Euroscarf | |
| YL515 | [BY4741/2] *MATα*; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | |
| YL516 | [BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | 17A-C; 18A-B; 19A-C |
| MB1537 | [YL516] *MAT***a** *gtr1Δ::kanMX4* | (Binda *et al.*, 2009) | 17A, C; 18A; 19A-B |

**Strains used in ChapterIII**

| Strain | Genotype | Source or Reference | Figure |
|---|---|---|---|
| EGY48 | *MATα his3 trp1 ura3 LEU2::LexAop6-LEU2* (+pSH18-34) | (Zervos *et al.*, 1993) | Table 4; Table 5; 20 |
| FD35 | [KT1960] *MAT***a** *ura3 leu2 his3 trp1 TCO89-TAP::HIS3* | (Binda *et al.*, 2009) | 21 |
| BY4741 | *MAT***a**; *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* | Euroscarf | |
| BY4742 | *MATα*; *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* | Euroscarf | |
| Y08149 | [BY4741] *MAT***a**; $cdc60^{ts}$ | (Li *et al.*, 2011) | 27B |
| YL515 | [BY4741/2] *MATα*; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | |
| YL516 | [BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0* | (Binda *et al.*, 2009) | Table 7; 25A-C; 28A-D; 29A; 30B, 32 |
| GB2381 | [YL516] *MAT***a**; $CDC60$-$HA_3$*::HIS3* | This study | 23A-C; 26A, B, D; |
| GB2382 | [YL516] *MAT***a**; *IGO1-TAP-kanMX4,* $CDC60$-$HA_3$*-HIS3* | This study | Table 7; 23A, C |
| GB2549 | [YL516] *MAT***a**; $CDC60$-$HA_3$*-HIS3, gtr2Δ::kanMX4* | (Bonfils *et al.*, 2012) | 23C |
| GB2523 | [YL516] *MAT***a**; *KOG1-HA::kanMX4* | (Bonfils *et al.*, 2012) | 26C |
| MPG1630 | [YL516] *MAT***a**; *gtr1Δ::kanMX4*, *gtr2Δ::kanMX4* | (Bonfils *et al.*, 2012) | 25A-C; 28B-C; 30A |
| GB2378 | [YL516] *MAT***a**; $cdc60^{D418R}$ | (Bonfils *et al.*, 2012) | 25A-C |
| GB2379 | [YL516] *MAT***a**; $cdc60^{D418R}$- $HA_3$*::HIS3* | (Bonfils *et al.*, 2012) | 26B |
| MB32 | [YL516] *MAT***a**; *gtr1Δ::kanMX4* | (Binda *et al.*, 2009) | 30A |
| MJA2638 | [YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-*CDC60*] | (Bonfils *et al.*, 2012) | 29B; 30A |
| MJA2786 | [YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-$CDC60^{D419A}$] | (Bonfils *et al.*, 2012) | 29B |
| MJA2784 | [YL515] *MATα; cdc60Δ::KanMX4* [*CEN, HIS3, CDC60*] | (Bonfils *et al.*, 2012) | 27A; 29B |
| MJA2785 | [YL515] *MATα; cdc60Δ::KanMX4* [*CEN, HIS3,* $CDC60^{D419A}$] | (Bonfils *et al.*, 2012) | 27A; 29B |
| MJA2604 | [YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-$cdc60^{S414F}$] | (Bonfils *et al.*, 2012) | 30A |
| 8003 | *MATα*; *leu2Δ0, ura3, trp1, his3, ade8,* $cdc60^{ts}$ | (Hohmann and Thevelein, 1992) | 32A-B |
| MPG2389 | *MAT***a**; *HIS3::GTR1-GFP, gtr1Δ::natMX4, ura3-52, leu2, trp1* | (Bonfils *et al.*, 2012) | 25D |
| MP52-2A | [YL516] *MAT***a**; *TOR1-D330-3xGFP* | (Binda *et al.*, 2009) | 25E |
| NMY51 | *MAT***a**; *his3Δ200, trp1-901, leu2-3,112, ade2, LYS::(lexAop)4-HIS3, ura3::(lexAop)8- lacZ, ade2::(lexAop)8-ADE2 GAL4* | Dualsystems | 31A; 34A-B |

**Plasmids used in Chapter I**

| Plasmid | Description | Source or Reference | Figure |
|---|---|---|---|
| pRS413 | *CEN, HIS3* | (Brachmann *et al.*, 1998) | 13; 14A-B |
| pRS416 | *CEN, URA3* | (Brachmann *et al.*, 1998) | 16A-C |
| pJU1436 | pRS416-$SCH9^{T570A}$-$HA_5$ | (Urban *et al.*, 2007) | 9C; 12B; 13; 14A-B |
| YCplac111 | *CEN, LEU2* | (Gietz and Sugino, 1988) | 14A-B; 16A-C |
| pJU1462 | pRS413-$SCH9^{T570A}$-$HA_5$ | (Urban *et al.*, 2007) | 16A-C |

**Plasmids used in Chapter II**

| Plasmid | Description | Source or Reference | Figure |
|---|---|---|---|
| pJU1462 | pRS413-$SCH9^{T570A}$-$HA_5$ | (Urban *et al.*, 2007) | 17A-C; 18A-B; 19A-C |
| YCplac33 | *CEN, URA3* | (Gietz and Sugino, 1988) | 17A-C; 18A-B; 19A-C |
| pMB1394 | YCplac33-$Tet_{ON}$-*GTR1* | (Binda *et al.*, 2009) | 17A; 18A; 19A |
| pMB1394 | YCplac33-$Tet_{ON}$-$GTR1^{Q65L}$ | (Binda *et al.*, 2009) | 17A, C; 18A; 19A, B |
| pMB1395 | YCplac33-$Tet_{ON}$-$GTR1^{S20L}$ | (Binda *et al.*, 2009) | 17A; 18A; 19A |

**Plasmids used in Chapter III**

| Plasmid | Description | Source or Reference | Figure |
|---|---|---|---|
| pJG4-5 | *2μ, TRP1 GAL1-HA-LexA*$^{AD}$ | (Gyuris *et al.*, 1993) | |
| pEG202 | *2μ, HIS3 ADH1-LexA*$^{DBD}$ | (Gyuris *et al.*, 1993) | 20 |
| pFD1118 | pEG202-*ADH1-GTR1* | | Table 4, Table 5, 20 |
| pCDV1248 | pEG202-*ADH1-GTR1*$^{Q65L}$ | | Table 5, 20 |
| pCDV1249 | pEG202-*ADH1-GTR1*$^{S20L}$ | | Table 5, 20 |
| pCDV927 | pJG4-5-*GAL1-HA-GTR2* | | Table 5, 20 |
| pGB01 | pJG4-5-*GAL1-HA-BRF1* | | Table 5, 20 |
| p573 | pAU5-*FPR1-GST* | | 21 |
| pGEX-3X | *AmpR*, *GST* (for bacterial expression) | (Smith and Johnson, 1988) | |
| pGB1792 | pGEX-3X-*BRF1* | | 21, 22 |
| pHAC33 | *CEN, URA3, HA* | | |
| pRL1095 | pHAC33-*SCH9-HA*$_3$ | | 22 |
| pRL1096 | pHAC33-*SCH9-K441A-HA*$_3$ | | 22 |
| YCplac33 | *CEN, URA3* | (Gietz and Sugino, 1988) | 25A-C, 28A-D, 30A |
| YCplac111 | *CEN, LEU2* | (Gietz and Sugino, 1988) | 25A-C, 28A-C, 30A |
| pMB1344 | YCplac33-*GTR1-TAP* | (Binda *et al.*, 2009) | Table 7, 23A-C, 25A-B, 31B |
| pCM264 | *CEN, URA3, Tet*$_{OFF}$*-HIS*$_6$*-HA*$_3$ | (Arino and Herrero, 2003) | 24A-B, 27B |
| pGB1957 | pCM264-*Tet*$_{OFF}$*-HIS*$_6$*-CDC60* | This study | 24A-B, 27B |
| pJU1462 | pRS413-*SCH9*$^{T570A}$*-HA*$_5$ | (Urban *et al.*, 2007) | 24A-B, 27A-B, 28A-D, 29A, 32 |
| pMB1394 | YCplac33-*Tet*$_{ON}$*-GTR1*$^{Q65L}$ | (Binda *et al.*, 2009) | 25A-C, 28B-C, 30A |
| pPM1623 | YCplac111-*Tet*$_{ON}$*-GTR2*$^{S23L}$ | This study | 25A-C, 28B-C, 30A |
| pMJ1974 | YCplac111-*CDC60* | This study | 29A-B, 30A |
| pMJ2113 | YCplac111-*CDC60*$^{S414F}$ | This study | 30A |
| pDL2-Alg5 | 2μ, *ADH1-HA-NubG, TRP1* | Dualsystems | 31A |
| pAI-Alg5 | 2μ, *ADH1-HA-NubI,TRP1* | Dualsystems | 31A |
| pPR3-N | 2μ, *CYC1-NubG-HA, TRP1* | Dualsystems | 31A |
| pNP1689 | pPR3-N-*CYC1-NubG-HA-GTR1* | (Binda *et al.*, 2009) | 31A, 34A-B |
| pNP1692 | pPR3-N-*CYC1-NubG-HA-GTR2* | (Binda *et al.*, 2009) | 31A, 34A-B |
| pMJ1868[1] | pCabWT-*CYC1-Cub-LexA-CDC60*$^{CP1}$ | This study | 31A, 34A-B |
| pMJ2115[1] | pCabWT-*CYC1-Cub-LexA-CDC60*$^{CP1-S414F}$ | This study | 31A |
| pMPG1574 | 2μ, *Tet*$_{ON}$*-HIS*$_6$*-HA*$_3$*, URA3* | (Binda *et al.*, 2009) | 31B, 32 |
| pMJ2059[1] | pMPG1574-*Tet*$_{ON}$*-HIS*$_6$*-HA*$_3$*-CDC60*$^{CP1}$ | This study | 31B, 32 |
| pMJ2116[1] | pMPG1574-*Tet*$_{ON}$*-HIS*$_6$*-HA*$_3$*-CDC60*$^{CP1-S414F}$ | This study | 31B, 32 |
| pMB1372 | *YCplac33- GTR1*$^{Q65L}$ *-TAP* | (Binda *et al.*, 2009) | 26D |
| pMJA2192 | pRS416-*CYC1-CDC60-RFP, URA3* | This study | 25D |
| pMJA2069 | YCplac111-*CDC60*$^{D419A}$ | This study | 29B |
| pJU1436 | pRS416-*SCH9*$^{T570A}$*-HA*$_5$ | (Urban *et al.*, 2007) | |
| pMJA2168 | *CEN, HIS3, CDC60* | This study | 27A, 29B |
| pMJA2167 | *CEN, HIS3, CDC60*$^{D419A}$ | This study | 27A, 29B |
| pMJA1906 | pCabWT-*CYC1-Cub-LexA-ILS1*$^{CP1}$ | | 34A-B |
| pMJA1908 | pCabWT-*CYC1-Cub-LexA-VAS1*$^{CP1}$ | | 34A-B |

# - References -

Abastado J.P., Miller P.F., Jackson B.M., Hinnebusch A.G. (1991). Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. *Mol Cell Biol* **11:** 486-496.
Abdel-Sater F., El Bakkoury M., Urrestarazu A., Vissers S., Andre B. (2004). Amino acid signaling in yeast: casein kinase I and the Ssy5 endoprotease are key determinants of endoproteolytic activation of the membrane-bound Stp1 transcription factor. *Mol Cell Biol* **24:** 9771-9785.
Abdel-Sater F., Jean C., Merhi A., Vissers S., Andre B. (2011). Amino acid signaling in yeast: activation of Ssy5 protease is associated with its phosphorylation-induced ubiquitylation. *J Biol Chem* **286:** 12006-12015.
Abeliovich H., Dunn W.A., Jr., Kim J., Klionsky D.J. (2000). Dissection of autophagosome biogenesis into distinct nucleation and expansion steps. *J Cell Biol* **151:** 1025-1034.
Adami A., García-Álvarez B., Arias-Palomo E., Barford D., Llorca O. (2007). Structure of TOR and Its Complex with KOG1. *Molecular Cell* **27:** 509-516.
Alzamora R., Thali R.F., Gong F., Smolak C., Li H., Baty C.J., Bertrand C.A., Auchli Y., Brunisholz R.A., Neumann D., Hallows K.R., Pastor-Soler N.M. (2010). PKA regulates vacuolar H+-ATPase localization and activity via direct phosphorylation of the a subunit in kidney cells. *J Biol Chem* **285:** 24676-24685.
Andrade M.A., Bork P. (1995). HEAT repeats in the Huntington's disease protein. *Nat Genet* **11:** 115-116.
Andrade M.A., Petosa C., O'Donoghue S.I., Muller C.W., Bork P. (2001). Comparison of ARM and HEAT protein repeats. *J Mol Biol* **309:** 1-18.
Andre B., Hein C., Grenson M., Jauniaux J.C. (1993). Cloning and expression of the UGA4 gene coding for the inducible GABA-specific transport protein of Saccharomyces cerevisiae. *Mol Gen Genet* **237:** 17-25.
Andreasson C., Ljungdahl P.O. (2002). Receptor-mediated endoproteolytic activation of two transcription factors in yeast. *Genes Dev* **16:** 3158-3172.
Anthony J.C., Anthony T.G., Kimball S.R., Vary T.C., Jefferson L.S. (2000). Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr* **130:** 139-145.
Antonellis A., Ellsworth R.E., Sambuughin N., Puls I., Abel A., Lee-Lin S.Q., Jordanova A., Kremensky I., Christodoulou K., Middleton L.T., Sivakumar K., Ionasescu V., Funalot B., Vance J.M., Goldfarb L.G., Fischbeck K.H., Green E.D. (2003). Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth disease type 2D and distal spinal muscular atrophy type V. *Am J Hum Genet* **72:** 1293-1299.
Araki T., Uesono Y., Oguchi T., Toh E.A. (2005). LAS24/KOG1, a component of the TOR complex 1 (TORC1), is needed for resistance to local anesthetic tetracaine and normal distribution of actin cytoskeleton in yeast. *Genes Genet Syst* **80:** 325-343.
Arino J., Herrero E. (2003). Use of tetracycline-regulatable promoters for functional analysis of protein phosphatases in yeast. *Methods Enzymol* **366:** 347-358.
Ataide S.F., Ibba M. (2006). Small molecules: big players in the evolution of protein synthesis. *ACS Chem Biol* **1:** 285-297.
Audhya A., Loewith R., Parsons A.B., Gao L., Tabuchi M., Zhou H., Boone C., Hall M.N., Emr S.D. (2004). Genome-wide lethality screen identifies new PI4,5P2 effectors that regulate the actin cytoskeleton. *EMBO J* **23:** 3747-3757.
Avruch J., Long X., Ortiz-Vega S., Rapley J., Papageorgiou A., Dai N. (2009). Amino acid regulation of TOR complex 1. *Am J Physiol Endocrinol Metab* **296:** E592-602.
Baars T.L., Petri S., Peters C., Mayer A. (2007). Role of the V-ATPase in Regulation of the Vacuolar Fission Fusion Equilibrium. *Molecular Biology of the Cell* **18:** 3873-3882.
Balgi A.D., Fonseca B.D., Donohue E., Tsang T.C., Lajoie P., Proud C.G., Nabi I.R., Roberge M. (2009). Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling. *PLoS One* **4:** e7124.
Barbet N.C., Schneider U., Helliwell S.B., Stansfield I., Tuite M.F., Hall M.N. (1996). TOR controls translation initiation and early G1 progression in yeast. *MBoC* **7:** 25-42.
Beck T., Hall M.N. (1999). The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **402:** 689-692.

Berset C., Trachsel H., Altmann M. (1998). The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **95:** 4264-4269.
Bertram P.G., Choi J.H., Carvalho J., Ai W., Zeng C., Chan T.F., Zheng X.F. (2000). Tripartite regulation of Gln3p by TOR, Ure2p, and phosphatases. *J Biol Chem* **275:** 35727-35733.
Binda M., Péli-Gulli M.-P., Bonfils G., Panchaud N., Urban J., Sturgill T.W., Loewith R., De Virgilio C. (2009). The Vam6 GEF Controls TORC1 by Activating the EGO Complex. *Molecular Cell* **35:** 563-573.
Blinder D., Coschigano P.W., Magasanik B. (1996). Interaction of the GATA factor Gln3p with the nitrogen regulator Ure2p in Saccharomyces cerevisiae. *J Bacteriol* **178:** 4734-4736.
Boban M., Zargari A., Andreasson C., Heessen S., Thyberg J., Ljungdahl P.O. (2006). Asi1 is an inner nuclear membrane protein that restricts promoter access of two latent transcription factors. *J Cell Biol* **173:** 695-707.
Bonfils G., Jaquenoud M., Bontron S., Ostrowicz C., Ungermann C., De Virgilio C. (2012). Leucyl-tRNA Synthetase Controls TORC1 via the EGO Complex. *Mol Cell*.
Boniecki M.T., Vu M.T., Betha A.K., Martinis S.A. (2008). CP1-dependent partitioning of pretransfer and posttransfer editing in leucyl-tRNA synthetase. *Proc Natl Acad Sci U S A* **105:** 19223-19228.
Boucherie H. (1985). Protein synthesis during transition and stationary phases under glucose limitation in Saccharomyces cerevisiae. *J Bacteriol* **161:** 385-392.
Bowers K., Stevens T.H. (2005). Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. *Biochim Biophys Acta* **1744:** 438-454.
Brachmann C.B., Davies A., Cost G.J., Caputo E., Li J., Hieter P., Boeke J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14:** 115-132.
Breitkreutz A., Choi H., Sharom J.R., Boucher L., Neduva V., Larsen B., Lin Z.Y., Breitkreutz B.J., Stark C., Liu G., Ahn J., Dewar-Darch D., Reguly T., Tang X., Almeida R., Qin Z.S., Pawson T., Gingras A.C., Nesvizhskii A.I., Tyers M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328:** 1043-1046.
Budovskaya Y.V., Stephan J.S., Deminoff S.J., Herman P.K. (2005). An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **102:** 13933-13938.
Bun-Ya M., Harashima S., Oshima Y. (1992). Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in Saccharomyces cerevisiae. *Mol Cell Biol* **12:** 2958-2966.
Burnett P.E., Barrow R.K., Cohen N.A., Snyder S.H., Sabatini D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* **95:** 1432-1437.
Buse M.G., Reid S.S. (1975). Leucine. A possible regulator of protein turnover in muscle. *J Clin Invest* **56:** 1250-1261.
Buse M.G., Atwell R., Mancusi V. (1979). In vitro effect of branched chain amino acids on the ribosomal cycle in muscles of fasted rats. *Horm Metab Res* **11:** 289-292.
Butow R.A., Avadhani N.G. (2004). Mitochondrial signaling: the retrograde response. *Mol Cell* **14:** 1-15.
Cagney G., Uetz P., Fields S. (2000). High-throughput screening for protein-protein interactions using two-hybrid assay. In: Jeremy Thorner S.D.E., John N.A. (eds). *Methods in Enzymology*. Academic Press. pp 3-14.
Cameroni E., Hulo N., Roosen J., Winderickx J., De Virgilio C. (2004). The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms. *Cell Cycle* **3:** 462-468.
Cardenas M., Cutler S.N., Lorenz M.C., Di Como C.J., Heitman J. (1999). The TOR signaling cascade regulates gene expression in response to nutrients. *Genes & Development*.
Cardenas M.E., Heitman J. (1995). FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. *EMBO J* **14:** 5892-5907.
Chedin S., Laferte A., Hoang T., Lafontaine D.L., Riva M., Carles C. (2007). Is ribosome synthesis controlled by pol I transcription? *Cell Cycle* **6:** 11-15.

Chen E.J., Kaiser C.A. (2003). LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. *J Cell Biol* **161:** 333-347.
Chen X., Ma J.J., Tan M., Yao P., Hu Q.H., Eriani G., Wang E.D. (2011). Modular pathways for editing non-cognate amino acids by human cytoplasmic leucyl-tRNA synthetase. *Nucleic Acids Res* **39:** 235-247.
Chen Y., Klionsky D.J. (2011). The regulation of autophagy - unanswered questions. *J Cell Sci* **124:** 161-170.
Cherkasova V.A., Hinnebusch A.G. (2003). Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes & Development* **17:** 859-872.
Choi J.W., Kim D.G., Park M.C., Um J.Y., Han J.M., Park S.G., Choi E.C., Kim S. (2009). AIMP2 promotes TNFalpha-dependent apoptosis via ubiquitin-mediated degradation of TRAF2. *J Cell Sci* **122:** 2710-2715.
Christie G.R., Hajduch E., Hundal H.S., Proud C.G., Taylor P.M. (2002). Intracellular Sensing of Amino Acids in Xenopus laevis Oocytes Stimulates p70 S6 Kinase in a Target of Rapamycin-dependent Manner. *Journal of Biological Chemistry* **277:** 9952-9957.
Coffman J.A., Rai R., Cooper T.G. (1995). Genetic evidence for Gln3p-independent, nitrogen catabolite repression-sensitive gene expression in Saccharomyces cerevisiae. *J Bacteriol* **177:** 6910-6918.
Coffman J.A., Rai R., Loprete D.M., Cunningham T., Svetlov V., Cooper T.G. (1997). Cross regulation of four GATA factors that control nitrogen catabolic gene expression in Saccharomyces cerevisiae. *J Bacteriol* **179:** 3416-3429.
Cooper T.G., Lam C., Turoscy V. (1980). Structural analysis of the dur loci in S. cerevisiae: two domains of a single multifunctional gene. *Genetics* **94:** 555-580.
Cooper T.G. (1982). *Nitrogen Metabolism in Saccharomyces cerevisiae*.
Cosentino G.P., Schmelzle T., Haghighat A., Helliwell S.B., Hall M.N., Sonenberg N. (2000). Eap1p, a novel eukaryotic translation initiation factor 4E-associated protein in Saccharomyces cerevisiae. *Mol Cell Biol* **20:** 4604-4613.
Courchesne W.E., Magasanik B. (1988). Regulation of nitrogen assimilation in Saccharomyces cerevisiae: roles of the URE2 and GLN3 genes. *J Bacteriol* **170:** 708-713.
Cox K.H., Kulkarni A., Tate J.J., Cooper T.G. (2004). Gln3 phosphorylation and intracellular localization in nutrient limitation and starvation differ from those generated by rapamycin inhibition of Tor1/2 in Saccharomyces cerevisiae. *J Biol Chem* **279:** 10270-10278.
Crespo J.L., T. P., B. F., Hall M.N. (2002). The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. *Proceedings of the National Academy of Sciences* **99:** 6784-6789.
Cusack S., Berthet-Colominas C., Hartlein M., Nassar N., Leberman R. (1990). A second class of synthetase structure revealed by X-ray analysis of Escherichia coli seryl-tRNA synthetase at 2.5 A. *Nature* **347:** 249-255.
Cytrynska M., Frajnt M., Jakubowicz T. (2001). Saccharomyces cerevisiae pyruvate kinase Pyk1 is PKA phosphorylation substrate in vitro. *FEMS Microbiol Lett* **203:** 223-227.
Dalley J.A., Selkirk A., Pool M.R. (2008). Access to ribosomal protein Rpl25p by the signal recognition particle is required for efficient cotranslational translocation. *Mol Biol Cell* **19:** 2876-2884.
Darsow T., Rieder S.E., Emr S.D. (1997). A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J Cell Biol* **138:** 517-529.
Dasgupta A., Darst R.P., Martin K.J., Afshari C.A., Auble D.T. (2002). Mot1 activates and represses transcription by direct, ATPase-dependent mechanisms. *Proc Natl Acad Sci U S A* **99:** 2666-2671.
Davis R.H. (1972). Metabolite distribution in cells. *Science* **178:** 835-840.
de Boer M., Nielsen P.S., Bebelman J.P., Heerikhuizen H., Andersen H.A., Planta R.J. (2000). Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of Saccharomyces cerevisiae. *Nucleic Acids Res* **28:** 974-981.
De Craene J.O., Soetens O., Andre B. (2001). The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. *J Biol Chem* **276:** 43939-43948.
De Virgilio C., Loewith R. (2006). Cell growth control: little eukaryotes make big contributions. *Oncogene* **25:** 6392-6415.

Delarue M., Moras D. (1993). The aminoacyl-tRNA synthetase family: modules at work. *Bioessays* **15:** 675-687.
Delforge J., Messenguy F., Wiame J.M. (1975). The regulation of arginine biosynthesis in Saccharomyces cerevisiae. The specificity of argR- mutations and the general control of amino-acid biosynthesis. *Eur J Biochem* **57:** 231-239.
Dennis M.D., Baum J.I., Kimball S.R., Jefferson L.S. (2011). Mechanisms Involved in the Coordinate Regulation of mTORC1 by Insulin and Amino Acids. *Journal of Biological Chemistry* **286:** 8287-8296.
DeRisi J.L., Iyer V.R., Brown P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278:** 680-686.
Desrivieres S., Cooke F.T., Parker P.J., Hall M.N. (1998). MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in Saccharomyces cerevisiae. *J Biol Chem* **273:** 15787-15793.
Di Como C.J., Arndt K.T. (1996). Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev* **10:** 1904-1916.
Didion T., Regenberg B., Jorgensen M.U., Kielland-Brandt M.C., Andersen H.A. (1998). The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in Saccharomyces cerevisiae. *Mol Microbiol* **27:** 643-650.
Dihazi H., Kessler R., Eschrich K. (2003). Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. *Biochemistry* **42:** 6275-6282.
Dilova I., Aronova S., Chen J.C., Powers T. (2004). Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1.Rtg3p-dependent target genes. *J Biol Chem* **279:** 46527-46535.
Dong J., Qiu H., Garcia-Barrio M., Anderson J., Hinnebusch A.G. (2000). Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell* **6:** 269-279.
Drose S., Bindseil K.U., Bowman E.J., Siebers A., Zeeck A., Altendorf K. (1993). Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. *Biochemistry* **32:** 3902-3906.
Dubouloz F., Deloche O., Wanke V., Cameroni E., De Virgilio C. (2005). The TOR and EGO Protein Complexes Orchestrate Microautophagy in Yeast. *Molecular Cell* **19:** 15-26.
Dupont N., Temime-Smaali N., Lafont F. (2010). How ubiquitination and autophagy participate in the regulation of the cell response to bacterial infection. *Biol Cell* **102:** 621-634.
Duvel K., Santhanam A., Garrett S., Schneper L., Broach J.R. (2003). Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. *Mol Cell* **11:** 1467-1478.
Echols N., Harrison P., Balasubramian S., Luscombe L.S., Bertone P., Zhang Z., M. G. (2002). A comprehensive analysis of amno acid and nucleotide composition in eukaryotic genomes, comparing genes and pseudogenes. *Nucleic Acid Research* **30:** 2515-2523.
Edinger A.L., Thompson C.B. (2002). Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell* **13:** 2276-2288.
Edinger A.L., Linardic C.M., Chiang G.G., Thompson C.B., Abraham R.T. (2003). Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. *Cancer Res* **63:** 8451-8460.
Epple U.D., Suriapranata I., Eskelinen E.L., Thumm M. (2001). Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *J Bacteriol* **183:** 5942-5955.
Escobar-Henriques M. (2001). Proteome Analysis and Morphological Studies Reveal Multiple Effects of the Immunosuppressive Drug Mycophenolic Acid Specifically Resulting from Guanylic Nucleotide Depletion. *Journal of Biological Chemistry* **276:** 46237-46242.
Fabrizio P., Pozza F., Pletcher S.D., Gendron C.M., Longo V.D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292:** 288-290.
Fairley J.A., Scott P.H., White R.J. (2003). TFIIIB is phosphorylated, disrupted and selectively released from tRNA promoters during mitosis in vivo. *EMBO J* **22:** 5841-5850.

Fairley J.A., Mitchell L.E., Berg T., Kenneth N.S., von Schubert C., Sillje H.H., Medema R.H., Nigg E.A., White R.J. (2012). Direct Regulation of tRNA and 5S rRNA Gene Transcription by Polo-like Kinase 1. *Mol Cell* **45:** 541-552.
Fairweather I., Boray J.C. (1999). Fasciolicides: efficacy, actions, resistance and its management. *Vet J* **158:** 81-112.
Felton-Edkins Z.A., Fairley J.A., Graham E.L., Johnston I.M., White R.J., Scott P.H. (2003). The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. *EMBO J* **22:** 2422-2432.
Fersht A.R., Kaethner M.M. (1976). Enzyme hyperspecificity. Rejection of threonine by the valyl-tRNA synthetase by misacylation and hydrolytic editing. *Biochemistry* **15:** 3342-3346.
Fersht A.R. (1977). Editing mechanisms in protein synthesis. Rejection of valine by the isoleucyl-tRNA synthetase. *Biochemistry* **16:** 1025-1030.
Fischer von Mollard G., Stevens T.H. (1999). The Saccharomyces cerevisiae v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole. *Mol Biol Cell* **10:** 1719-1732.
Fonseca B.D., Smith E.M., Lee V.H., MacKintosh C., Proud C.G. (2007). PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. *J Biol Chem* **282:** 24514-24524.
Fonseca B.D., Diering G.H., Bidinosti M.A., Dalal K., Alain T., Balgi A.D., Forestieri R., Nodwell M., Rajadurai C.V., Gunaratnam C., Tee A.R., Duong F., Andersen R.J., Orlowski J., Numata M., Sonenberg N., Roberge M. (2012). Structure-activity analysis of niclosamide reveals a potential role for cytoplasmic pH in the control of mammalian target of rapamycin complex 1 (mTORC1) signaling. *J Biol Chem*.
Forgac M. (2007). Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol* **8:** 917-929.
Forsberg H., Hammar M., Andreasson C., Moliner A., Ljungdahl P.O. (2001). Suppressors of ssy1 and ptr3 null mutations define novel amino acid sensor-independent genes in Saccharomyces cerevisiae. *Genetics* **158:** 973-988.
Forsberg H., Ljungdahl P.O. (2001). Sensors of extracellular nutrients in Saccharomyces cerevisiae. *Curr Genet* **40:** 91-109.
Forsburg S.L. (2001). The art and design of genetic screens: yeast. *Nat Rev Genet* **2:** 659-668.
Fox H.L., Pham P.T., Kimball S.R., Jefferson L.S., Lynch C.J. (1998). Amino acid effects on translational repressor 4E-BP1 are mediated primarily by L-leucine in isolated adipocytes. *Am J Physiol* **275:** C1232-1238.
Franklin T.J., Cook J.M. (1969). The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochem J* **113:** 515-524.
Gaber R.F., Ottow K., Andersen H.A., Kielland-Brandt M.C. (2003). Constitutive and hyperresponsive signaling by mutant forms of Saccharomyces cerevisiae amino acid sensor Ssy1. *Eukaryot Cell* **2:** 922-929.
Gallego C., Gari E., Colomina N., Herrero E., Aldea M. (1997). The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. *EMBO J* **16:** 7196-7206.
Gao M., Kaiser C.A. (2006). A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. *Nat Cell Biol* **8:** 657-667.
Gao X.D., Wang J., Keppler-Ross S., Dean N. (2005). ERS1 encodes a functional homologue of the human lysosomal cystine transporter. *FEBS J* **272:** 2497-2511.
Gelperin D.M., White M.A., Wilkinson M.L., Kon Y., Kung L.A., Wise K.J., Lopez-Hoyo N., Jiang L., Piccirillo S., Yu H., Gerstein M., Dumont M.E., Phizicky E.M., Snyder M., Grayhack E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Dev* **19:** 2816-2826.
Ghaemmaghami S., Huh W.K., Bower K., Howson R.W., Belle A., Dephoure N., O'Shea E.K., Weissman J.S. (2003). Global analysis of protein expression in yeast. *Nature* **425:** 737-741.
Giannattasio S., Liu Z., Thornton J., Butow R.A. (2005). Retrograde response to mitochondrial dysfunction is separable from TOR1/2 regulation of retrograde gene expression. *J Biol Chem* **280:** 42528-42535.

Gietz R.D., Sugino A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527-534.
Godard P., Urrestarazu A., Vissers S., Kontos K., Bontempi G., van Helden J., Andre B. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* **27:** 3065-3086.
Gong R., Li L., Liu Y., Wang P., Yang H., Wang L., Cheng J., Guan K.L., Xu Y. (2011). Crystal structure of the Gtr1p-Gtr2p complex reveals new insights into the amino acid-induced TORC1 activation. *Genes & Development* **25:** 1668-1673.
Gonzalez I.M., Martin P.M., Burdsal C., Sloan J.L., Mager S., Harris T., Sutherland A.E. (2012). Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. *Dev Biol* **361:** 286-300.
Gromova I., Celis J.E. (2006). Chapter 27 - Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass Spectrometry. In: Julio E.C. (ed). *Cell Biology (Third Edition)*. Academic Press: Burlington. pp 219-223.
Guertin D.A., Sabatini D.M. (2007). Defining the role of mTOR in cancer. *Cancer Cell* **12:** 9-22.
Guthrie C., Fink G.R. (2002). *Guide to Yeast Genetics and Molecular and Cell Biology*. Academic Press.
Gyuris J., Golemis E., Chertkov H., Brent R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75:** 791-803.
Han J.M., Park S.G., Lee Y., Kim S. (2006). Structural separation of different extracellular activities in aminoacyl-tRNA synthetase-interacting multi-functional protein, p43/AIMP1. *Biochem Biophys Res Commun* **342:** 113-118.
Han J.M., Park B.J., Park S.G., Oh Y.S., Choi S.J., Lee S.W., Hwang S.K., Chang S.H., Cho M.H., Kim S. (2008). AIMP2/p38, the scaffold for the multi-tRNA synthetase complex, responds to genotoxic stresses via p53. *Proc Natl Acad Sci U S A* **105:** 11206-11211.
Han J.M., Jeong S.J., Park M.C., Kim G., Kwon N.H., Kim H.K., Ha S.H., Ryu S.H., Kim S. (2012). Leucyl-tRNA Synthetase Is an Intracellular Leucine Sensor for the mTORC1-Signaling Pathway. *Cell*.
Hara K., Maruki Y., Long X., Yoshino K., Oshiro N., Hidayat S., Tokunaga C., Avruch J., Yonezawa K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110:** 177-189.
Hardwick J.S., Kuruvilla F.G., Tong J.K., Shamji A.F., Schreiber S.L. (1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc Natl Acad Sci U S A* **96:** 14866-14870.
Heitman J., Movva N.R., Hall M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253:** 905-909.
Hemmings B.A., Adams-Pearson C., Maurer F., Muller P., Goris J., Merlevede W., Hofsteenge J., Stone S.R. (1990). alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* **29:** 3166-3173.
Heublein S., Kazi S., Ögmundsdóttir M.H., Attwood E.V., Kala S., Boyd C.A.R., Wilson C., Goberdhan D.C.I. (2010). Proton-assisted amino-acid transporters are conserved regulators of proliferation and amino-acid-dependent mTORC1 activation. *Oncogene* **29:** 4068-4079.
Hinnebusch A.G. (1984). Evidence for translational regulation of the activator of general amino acid control in yeast. *Proc Natl Acad Sci U S A* **81:** 6442-6446.
Hinnebusch A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* **59:** 407-450.
Hirakata M., Suwa A., Nagai S., Kron M.A., Trieu E.P., Mimori T., Akizuki M., Targoff I.N. (1999). Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. *J Immunol* **162:** 2315-2320.
Hofman-Bang J. (1999). Nitrogen catabolite repression in Saccharomyces cerevisiae. *Mol Biotechnol* **12:** 35-73.
Hohmann S., Thevelein J.M. (1992). The cell division cycle gene CDC60 encodes cytosolic leucyl-tRNA synthetase in Saccharomyces cerevisiae. *Gene* **120:** 43-49.

Holst B., Lunde C., Lages F., Oliveira R., Lucas C., Kielland-Brandt M.C. (2000). GUP1 and its close homologue GUP2, encoding multimembrane-spanning proteins involved in active glycerol uptake in Saccharomyces cerevisiae. *Mol Microbiol* **37:** 108-124.
Homma K., Terui S., Minemura M., Qadota H., Anraku Y., Kanaho Y., Ohya Y. (1998). Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. *J Biol Chem* **273:** 15779-15786.
Huber A., Bodenmiller B., Uotila A., Stahl M., Wanka S., Gerrits B., Aebersold R., Loewith R. (2009). Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. *Genes & Development* **23:** 1929-1943.
Huh W.K., Falvo J.V., Gerke L.C., Carroll A.S., Howson R.W., Weissman J.S., O'Shea E.K. (2003). Global analysis of protein localization in budding yeast. *Nature* **425:** 686-691.
Iiboshi Y., Papst P.J., Kawasome H., Hosoi H., Abraham R.T., Houghton P.J., Terada N. (1999). Amino acid-dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. *J Biol Chem* **274:** 1092-1099.
Inoki K., Guan K.L. (2006). Complexity of the TOR signaling network. *Trends Cell Biol* **16:** 206-212.
Iraqui I., Vissers S., Bernard F., de Craene J.O., Boles E., Urrestarazu A., Andre B. (1999). Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. *Mol Cell Biol* **19:** 989-1001.
Jacinto E., Guo B., Arndt K.T., Schmelzle T., Hall M.N. (2001). TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. *Mol Cell* **8:** 1017-1026.
Jakubowski H., Goldman E. (1992). Editing of errors in selection of amino acids for protein synthesis. *Microbiol Rev* **56:** 412-429.
Jakubowski H. (2011). Quality control in tRNA charging -- editing of homocysteine. *Acta Biochim Pol* **58:** 149-163.
Jauniaux J.C., Grenson M. (1990). GAP1, the general amino acid permease gene of Saccharomyces cerevisiae. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *Eur J Biochem* **190:** 39-44.
Jia M.H., Larossa R.A., Lee J.M., Rafalski A., Derose E., Gonye G., Xue Z. (2000). Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. *Physiol Genomics* **3:** 83-92.
Jiang Y., Broach J.R. (1999). Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. *EMBO J* **18:** 2782-2792.
Jordanova A., Irobi J., Thomas F.P., Van Dijck P., Meerschaert K., Dewil M., Dierick I., Jacobs A., De Vriendt E., Guergueltcheva V., Rao C.V., Tournev I., Gondim F.A., D'Hooghe M., Van Gerwen V., Callaerts P., Van Den Bosch L., Timmermans J.P., Robberecht W., Gettemans J., Thevelein J.M., De Jonghe P., Kremensky I., Timmerman V. (2006). Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. *Nat Genet* **38:** 197-202.
Jorgensen P., Nishikawa J.L., Breitkreutz B.J., Tyers M. (2002). Systematic identification of pathways that couple cell growth and division in yeast. *Science* **297:** 395-400.
Jorgensen P., Tyers M. (2004). How cells coordinate growth and division. *Curr Biol* **14:** R1014-1027.
Ju Q., Warner J.R. (1994). Ribosome synthesis during the growth cycle of Saccharomyces cerevisiae. *Yeast* **10:** 151-157.
Kamada Y., Funakoshi T., Shintani T., Nagano K., Ohsumi M., Ohsumi Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* **150:** 1507-1513.
Kamada Y., Yoshino K., Kondo C., Kawamata T., Oshiro N., Yonezawa K., Ohsumi Y. (2010). Tor directly controls the Atg1 kinase complex to regulate autophagy. *Mol Cell Biol* **30:** 1049-1058.
Kane P.M. (1995). Disassembly and reassembly of the yeast vacuolar H(+)-ATPase in vivo. *J Biol Chem* **270:** 17025-17032.
Kawai S., Urban J., Piccolis M., Panchaud N., De Virgilio C., Loewith R. (2011). Mitochondrial Genomic Dysfunction Causes Dephosphorylation of Sch9 in the Yeast Saccharomyces cerevisiae. *Eukaryotic Cell* **10:** 1367-1369.

Kim D.H., Sarbassov D.D., Ali S.M., King J.E., Latek R.R., Erdjument-Bromage H., Tempst P., Sabatini D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110:** 163-175.
Kim E., Goraksha-Hicks P., Li L., Neufeld T.P., Guan K.-L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. *Nature Cell Biology* **10:** 935-945.
Kim J., Guan K.-L. (2011). Amino Acid Signaling in TOR Activation. *Annual Review of Biochemistry* **80:** 1001-1032.
Kim M.J., Park B.J., Kang Y.S., Kim H.J., Park J.H., Kang J.W., Lee S.W., Han J.M., Lee H.W., Kim S. (2003). Downregulation of FUSE-binding protein and c-myc by tRNA synthetase cofactor p38 is required for lung cell differentiation. *Nat Genet* **34:** 330-336.
Kimball S.R., Shantz L.M., Horetsky R.L., Jefferson L.S. (1999). Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J Biol Chem* **274:** 11647-11652.
Kissova I., Deffieu M., Manon S., Camougrand N. (2004). Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem* **279:** 39068-39074.
Kitamoto K., Yoshizawa K., Oshumi Y., Anraku Y. (1988). Dynamic Aspects of Vacuolar and Cytosolic Amino Acid Pools of *Saccharomyces cerevisiae*. *Journal of Bacterology* **170:** 2683-2686.
Klasson H., Fink G.R., Ljungdahl P.O. (1999). Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. *Mol Cell Biol* **19:** 5405-5416.
Ko Y.G., Kang Y.S., Kim E.K., Park S.G., Kim S. (2000). Nucleolar localization of human methionyl-tRNA synthetase and its role in ribosomal RNA synthesis. *J Cell Biol* **149:** 567-574.
Ko Y.G., Kim E.Y., Kim T., Park H., Park H.S., Choi E.J., Kim S. (2001). Glutamine-dependent antiapoptotic interaction of human glutaminyl-tRNA synthetase with apoptosis signal-regulating kinase 1. *J Biol Chem* **276:** 6030-6036.
Kogan K., Spear E.D., Kaiser C.A., Fass D. (2010). Structural Conservation of Components in the Amino Acid Sensing Branch of the TOR Pathway in Yeast and Mammals. *Journal of Molecular Biology* **402:** 388-398.
Köhrer C., RajBhandary U.L. (2008). The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs. *Methods* **44:** 129-138.
Komeili A., Wedaman K.P., O'Shea E.K., T. P. (2000). Mechanism of Metabolic Control: Target of Rapamycin Signaling Links Nitrogen Quality to the Activity of the Rtg1 and Rtg3 Transcription Factors. *Journal of Cell Biology* **151:** 863-878.
Konno Y., Natsumeda Y., Nagai M., Yamaji Y., Ohno S., Suzuki K., Weber G. (1991). Expression of human IMP dehydrogenase types I and II in Escherichia coli and distribution in human normal lymphocytes and leukemic cell lines. *J Biol Chem* **266:** 506-509.
Korolchuk V.I., Saiki S., Lichtenberg M., Siddiqi F.H., Roberts E.A., Imarisio S., Jahreiss L., Sarkar S., Futter M., Menzies F.M., O'Kane C.J., Deretic V., Rubinsztein D.C. (2011). Lysosomal positioning coordinates cellular nutrient responses. *Nature Cell Biology* **13:** 453-460.
Kozminski K.G., Beven L., Angerman E., Tong A.H., Boone C., Park H.O. (2003). Interaction between a Ras and a Rho GTPase couples selection of a growth site to the development of cell polarity in yeast. *Mol Biol Cell* **14:** 4958-4970.
Kraakman L., Lemaire K., Ma P., Teunissen A.W., Donaton M.C., Van Dijck P., Winderickx J., de Winde J.H., Thevelein J.M. (1999). A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol Microbiol* **32:** 1002-1012.
Kraft C., Deplazes A., Sohrmann M., Peter M. (2008). Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat Cell Biol* **10:** 602-610.
Krasowska A., Chmielewska L., Adamski R., Luczynski J., Witek S., Sigler K. (2004). The sensitivity of yeast and yeast-like cells to new lysosomotropic agents. *Cellular and Molecular Biology Letters* **9:** 675-683.
Kulkarni A., Buford T.D., Rai R., Cooper T.G. (2006). Differing responses of Gat1 and Gln3 phosphorylation and localization to rapamycin and methionine sulfoximine treatment in Saccharomyces cerevisiae. *FEMS Yeast Res* **6:** 218-229.

Kyriacou S.V., Deutscher M.P. (2008). An important role for the multienzyme aminoacyl-tRNA synthetase complex in mammalian translation and cell growth. *Mol Cell* **29:** 419-427.
Laferte A., Favry E., Sentenac A., Riva M., Carles C., Chedin S. (2006). The transcriptional activity of RNA polymerase I is a key determinant for the level of all ribosome components. *Genes Dev* **20:** 2030-2040.
Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., Higgins D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23:** 2947-2948.
Lee J., Moir R.D., Willis I.M. (2009). Regulation of RNA polymerase III transcription involves SCH9-dependent and SCH9-independent branches of the target of rapamycin (TOR) pathway. *J Biol Chem* **284:** 12604-12608.
Lee J.W., Beebe K., Nangle L.A., Jang J., Longo-Guess C.M., Cook S.A., Davisson M.T., Sundberg J.P., Schimmel P., Ackerman S.L. (2006a). Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. *Nature* **443:** 50-55.
Lee S.W. (2004). Aminoacyl-tRNA synthetase complexes: beyond translation. *Journal of Cell Science* **117:** 3725-3734.
Lee Y.N., Nechushtan H., Figov N., Razin E. (2004). The function of lysyl-tRNA synthetase and Ap4A as signaling regulators of MITF activity in FcepsilonRI-activated mast cells. *Immunity* **20:** 145-151.
Lee Y.S., Han J.M., Kang T., Park Y.I., Kim H.M., Kim S. (2006b). Antitumor activity of the novel human cytokine AIMP1 in an in vivo tumor model. *Mol Cells* **21:** 213-217.
Lee Y.S., Han J.M., Son S.H., Choi J.W., Jeon E.J., Bae S.C., Park Y.I., Kim S. (2008). AIMP1/p43 downregulates TGF-beta signaling via stabilization of smurf2. *Biochem Biophys Res Commun* **371:** 395-400.
Lefebvre-Legendre L., Balguerie A., Duvezin-Caubet S., Giraud M.F., Slonimski P.P., Di Rago J.P. (2003). F1-catalysed ATP hydrolysis is required for mitochondrial biogenesis in Saccharomyces cerevisiae growing under conditions where it cannot respire. *Mol Microbiol* **47:** 1329-1339.
Lei E.P., Stern C.A., Fahrenkrog B., Krebber H., Moy T.I., Aebi U., Silver P.A. (2003). Sac3 is an mRNA export factor that localizes to cytoplasmic fibrils of nuclear pore complex. *Mol Biol Cell* **14:** 836-847.
Lempiainen H., Uotila A., Urban J., Dohnal I., Ammerer G., Loewith R., Shore D. (2009). Sfp1 interaction with TORC1 and Mrs6 reveals feedback regulation on TOR signaling. *Mol Cell* **33:** 704-716.
Lenburg M.E., O'Shea E.K. (1996). Signaling phosphate starvation. *Trends Biochem Sci* **21:** 383-387.
Li H., Tsang C.K., Watkins M., Bertram P.G., Zheng X.F. (2006). Nutrient regulates Tor1 nuclear localization and association with rDNA promoter. *Nature* **442:** 1058-1061.
Li Z., Vizeacoumar F.J., Bahr S., Li J., Warringer J., Vizeacoumar F.S., Min R., Vandersluis B., Bellay J., Devit M., Fleming J.A., Stephens A., Haase J., Lin Z.Y., Baryshnikova A., Lu H., Yan Z., Jin K., Barker S., Datti A., Giaever G., Nislow C., Bulawa C., Myers C.L., Costanzo M., Gingras A.C., Zhang Z., Blomberg A., Bloom K., Andrews B., Boone C. (2011). Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nat Biotechnol* **29:** 361-367.
Lieber M., Smith B., Szakal A., Nelson-Rees W., Todaro G. (1976). A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* **17:** 62-70.
Ling J., Reynolds N., Ibba M. (2009). Aminoacyl-tRNA Synthesis and Translational Quality Control. *Annual Review of Microbiology* **63:** 61-78.
Liu Z., Butow R.A. (1999). A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. *Mol Cell Biol* **19:** 6720-6728.
Liu Z., Sekito T., Epstein C.B., Butow R.A. (2001). RTG-dependent mitochondria to nucleus signaling is negatively regulated by the seven WD-repeat protein Lst8p. *EMBO J* **20:** 7209-7219.
Ljungdahl P.O., Daignan-Fornier B. (2012). Regulation of Amino Acid, Nucleotide, and Phosphate Metabolism in Saccharomyces cerevisiae. *Genetics* **190:** 885-929.
Loewith R., Jacinto E., Wullschleger S., Lorberg A., Crespo J.L., Bonenfant D., Oppliger W., Jenoe P., Hall M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10:** 457-468.

Loftfield R.B., Vanderjagt D. (1972). The frequency of errors in protein biosynthesis. *Biochem J* **128:** 1353-1356.
Lynch C.J., Fox H.L., Vary T.C., Jefferson L.S., Kimball S.R. (2000). Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. *J Cell Biochem* **77:** 234-251.
MacGurn J.A., Hsu P.C., Smolka M.B., Emr S.D. (2011). TORC1 regulates endocytosis via Npr1-mediated phosphoinhibition of a ubiquitin ligase adaptor. *Cell* **147:** 1104-1117.
Magasanik B. (1992). *Regulation of Nitrogen Utilization*.
Magasanik B., Kaiser C.A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. *Gene* **290:** 1-18.
Marini A.M., Vissers S., Urrestarazu A., Andre B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter in Saccharomyces cerevisiae. *EMBO J* **13:** 3456-3463.
Marini A.M., Soussi-Boudekou S., Vissers S., Andre B. (1997). A family of ammonium transporters in Saccharomyces cerevisiae. *Mol Cell Biol* **17:** 4282-4293.
Martin D.E., Soulard A., Hall M.N. (2004). TOR regulates ribosomal protein gene expression via PKA and the Forkhead transcription factor FHL1. *Cell* **119:** 969-979.
Martinez P., Persson B.L. (1998). Identification, cloning and characterization of a derepressible Na+-coupled phosphate transporter in Saccharomyces cerevisiae. *Mol Gen Genet* **258:** 628-638.
Mathews M.B., Bernstein R.M. (1983). Myositis autoantibody inhibits histidyl-tRNA synthetase: a model for autoimmunity. *Nature* **304:** 177-179.
Mathews M.B., Reichlin M., Hughes G.R., Bernstein R.M. (1984). Anti-threonyl-tRNA synthetase, a second myositis-related autoantibody. *J Exp Med* **160:** 420-434.
May M.E., Buse M.G. (1989). Effects of branched-chain amino acids on protein turnover. *Diabetes Metab Rev* **5:** 227-245.
Mayer C., Zhao J., Yuan X., Grummt I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* **18:** 423-434.
Mellman I., Fuchs R., Helenius A. (1986). Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem* **55:** 663-700.
Messina E., Gazzaniga P., Micheli V., Barile L., Lupi F., Agliano A.M., Giacomello A. (2004). Low levels of mycophenolic acid induce differentiation of human neuroblastoma cell lines. *Int J Cancer* **112:** 352-354.
Miller P.F., Hinnebusch A.G. (1989). Sequences that surround the stop codons of upstream open reading frames in GCN4 mRNA determine their distinct functions in translational control. *Genes Dev* **3:** 1217-1225.
Mitchell A.P., Magasanik B. (1984). Regulation of glutamine-repressible gene products by the GLN3 function in Saccharomyces cerevisiae. *Mol Cell Biol* **4:** 2758-2766.
Miyaki M., Iijima T., Shiba K., Aki T., Kita Y., Yasuno M., Mori T., Kuroki T., Iwama T. (2001). Alterations of repeated sequences in 5' upstream and coding regions in colorectal tumors from patients with hereditary nonpolyposis colorectal cancer and Turcot syndrome. *Oncogene* **20:** 5215-5218.
Morath C., Zeier M. (2003). Review of the antiproliferative properties of mycophenolate mofetil in non-immune cells. *Int J Clin Pharmacol Ther* **41:** 465-469.
Mosch H.U., Scheier B., Lahti R., Mantsala P., Braus G.H. (1991). Transcriptional activation of yeast nucleotide biosynthetic gene ADE4 by GCN4. *J Biol Chem* **266:** 20453-20456.
Nadolski M.J., Linder M.E. (2009). Molecular recognition of the palmitoylation substrate Vac8 by its palmitoyltransferase Pfa3. *J Biol Chem* **284:** 17720-17730.
Nakashima N., Hayashi N., Noguchi E., Nishimoto T. (1996). Putative GTPase Gtr1p genetically interacts with the RanGTPase cycle in Saccharomyces cerevisiae. *J Cell Sci* **109 ( Pt 9):** 2311-2318.
Nakashima N., Noguchi E., Nishimoto T. (1999). Saccharomyces cerevisiae Putative G Protein, Gtr1p, Which Forms Complexes
With Itself and a Novel Protein Designated as Gtr2p, Negatively Regulates
the Ran/Gsp1p G Protein Cycle Through Gtr2p. *Genetics*: 853-867.
Nakatogawa H., Suzuki K., Kamada Y., Ohsumi Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol* **10:** 458-467.
Natarajan K., Meyer M.R., Jackson B.M., Slade D., Roberts C., Hinnebusch A.G., Marton M.J. (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21:** 4347-4368.

Nicklin P., Bergman P., Zhang B., Triantafellow E., Wang H., Nyfeler B., Yang H., Hild M., Kung C., Wilson C., Myer V.E., MacKeigan J.P., Porter J.A., Wang Y.K., Cantley L.C., Finan P.M., Murphy L.O. (2009). Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell* **136:** 521-534.
Niederberger P., Miozzari G., Hutter R. (1981). Biological role of the general control of amino acid biosynthesis in Saccharomyces cerevisiae. *Mol Cell Biol* **1:** 584-593.
Nishihara T., Akifusa S., Koseki T., Kato S., Muro M., Hanada N. (1995). Specific inhibitors of vacuolar-type $H^+$-ATPases in apoptotic cell death. *Biochemical and Biophysical Research Communications* **212:** 255-262.
Nordmann M., Cabrera M., Perz A., Brocker C., Ostrowicz C., Engelbrecht-Vandre S., Ungermann C. (2010). The Mon1-Ccz1 complex is the GEF of the late endosomal Rab7 homolog Ypt7. *Curr Biol* **20:** 1654-1659.
O'Donnell A.F., Apffel A., Gardner R.G., Cyert M.S. (2010). Alpha-arrestins Aly1 and Aly2 regulate intracellular trafficking in response to nutrient signaling. *Mol Biol Cell* **21:** 3552-3566.
Oaks A., Bidwell R.G.S. (1970). Compartmentation of intermediary metabolites. *Annu Rev Plant Physiol* **21:** 43-66.
Ohsumi Y., Kitamoto K., Anraku Y. (1988). Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *J Bacteriol* **170:** 2676-2682.
Oshima Y. (1997). The phosphatase system in Saccharomyces cerevisiae. *Genes Genet Syst* **72:** 323-334.
Oshiro N., Takahashi R., Yoshino K., Tanimura K., Nakashima A., Eguchi S., Miyamoto T., Hara K., Takehana K., Avruch J., Kikkawa U., Yonezawa K. (2007). The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. *J Biol Chem* **282:** 20329-20339.
Palomino A., Herrero P., Moreno F. (2006). Tpk3 and Snf1 protein kinases regulate Rgt1 association with Saccharomyces cerevisiae HXK2 promoter. *Nucleic Acids Res* **34:** 1427-1438.
Park B.J., Kang J.W., Lee S.W., Choi S.J., Shin Y.K., Ahn Y.H., Choi Y.H., Choi D., Lee K.S., Kim S. (2005a). The haploinsufficient tumor suppressor p18 upregulates p53 via interactions with ATM/ATR. *Cell* **120:** 209-221.
Park B.J., Oh Y.S., Park S.Y., Choi S.J., Rudolph C., Schlegelberger B., Kim S. (2006a). AIMP3 haploinsufficiency disrupts oncogene-induced p53 activation and genomic stability. *Cancer Res* **66:** 6913-6918.
Park S.G., Kang Y.S., Ahn Y.H., Lee S.H., Kim K.R., Kim K.W., Koh G.Y., Ko Y.G., Kim S. (2002). Dose-dependent biphasic activity of tRNA synthetase-associating factor, p43, in angiogenesis. *J Biol Chem* **277:** 45243-45248.
Park S.G., Kim H.J., Min Y.H., Choi E.C., Shin Y.K., Park B.J., Lee S.W., Kim S. (2005b). Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. *Proc Natl Acad Sci U S A* **102:** 6356-6361.
Park S.G., Shin H., Shin Y.K., Lee Y., Choi E.C., Park B.J., Kim S. (2005c). The novel cytokine p43 stimulates dermal fibroblast proliferation and wound repair. *Am J Pathol* **166:** 387-398.
Park S.G., Kang Y.S., Kim J.Y., Lee C.S., Ko Y.G., Lee W.J., Lee K.U., Yeom Y.I., Kim S. (2006b). Hormonal activity of AIMP1/p43 for glucose homeostasis. *Proc Natl Acad Sci U S A* **103:** 14913-14918.
Patti M.E., Brambilla E., Luzi L., Landaker E.J., Kahn C.R. (1998). Bidirectional modulation of insulin action by amino acids. *The Journal of Clinical Investigation* **101:** 1519-1529.
Pearce L.R., Komander D., Alessi D.R. (2010). The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* **11:** 9-22.
Pedruzzi I., Burckert N., Egger P., De Virgilio C. (2000). Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. *EMBO J* **19:** 2569-2579.
Pedruzzi I., Dubouloz F., Cameroni E., Wanke V., Roosen J., Winderickx J., De Virgilio C. (2003). TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. *Mol Cell* **12:** 1607-1613.

Pena-Llopis S., Vega-Rubin-de-Celis S., Schwartz J.C., Wolff N.C., Tran T.A., Zou L., Xie X.J., Corey D.R., Brugarolas J. (2011). Regulation of TFEB and V-ATPases by mTORC1. *EMBO J* **30:** 3242-3258.
Peterson T.R., Laplante M., Thoreen C.C., Sancak Y., Kang S.A., Kuehl W.M., Gray N.S., Sabatini D.M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* **137:** 873-886.
Pluta K., Lefebvre O., Martin N.C., Smagowicz W.J., Stanford D.R., Ellis S.R., Hopper A.K., Sentenac A., Boguta M. (2001). Maf1p, a negative effector of RNA polymerase III in Saccharomyces cerevisiae. *Mol Cell Biol* **21:** 5031-5040.
Portela P., Moreno S., Rossi S. (2006). Characterization of yeast pyruvate kinase 1 as a protein kinase A substrate, and specificity of the phosphorylation site sequence in the whole protein. *Biochem J* **396:** 117-126.
Poulsen P., Gaber R.F., Kielland-Brandt M.C. (2008). Hyper- and hyporesponsive mutant forms of the Saccharomyces cerevisiae Ssy1 amino acid sensor. *Mol Membr Biol* **25:** 164-176.
Powers T., Walter P. (1999). Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. *Mol Biol Cell* **10:** 987-1000.
Ptacek J., Devgan G., Michaud G., Zhu H., Zhu X., Fasolo J., Guo H., Jona G., Breitkreutz A., Sopko R., McCartney R.R., Schmidt M.C., Rachidi N., Lee S.J., Mah A.S., Meng L., Stark M.J., Stern D.F., De Virgilio C., Tyers M., Andrews B., Gerstein M., Schweitzer B., Predki P.F., Snyder M. (2005). Global analysis of protein phosphorylation in yeast. *Nature* **438:** 679-684.
Qiu H., Hu C., Dong J., Hinnebusch A.G. (2002). Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2. *Genes Dev* **16:** 1271-1280.
Radonjic M., Andrau J.C., Lijnzaad P., Kemmeren P., Kockelkorn T.T., van Leenen D., van Berkum N.L., Holstege F.C. (2005). Genome-wide analyses reveal RNA polymerase II located upstream of genes poised for rapid response upon S. cerevisiae stationary phase exit. *Mol Cell* **18:** 171-183.
Ray P.S., Arif A., Fox P.L. (2007). Macromolecular complexes as depots for releasable regulatory proteins. *Trends Biochem Sci* **32:** 158-164.
Reinders J., Zahedi R.P., Pfanner N., Meisinger C., Sickmann A. (2006). Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res* **5:** 1543-1554.
Reinke A., Anderson S., McCaffery J.M., Yates J., 3rd, Aronova S., Chu S., Fairclough S., Iverson C., Wedaman K.P., Powers T. (2004). TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. *J Biol Chem* **279:** 14752-14762.
Rho S.B., Kim M.J., Lee J.S., Seol W., Motegi H., Kim S., Shiba K. (1999). Genetic dissection of protein-protein interactions in multi-tRNA synthetase complex. *Proc Natl Acad Sci U S A* **96:** 4488-4493.
Rhoads J.M., Corl B.A., Harrell R., Niu X., Gatlin L., Phillips O., Blikslager A., Moeser A., Wu G., Odle J. (2007). Intestinal ribosomal p70(S6K) signaling is increased in piglet rotavirus enteritis. *Am J Physiol Gastrointest Liver Physiol* **292:** G913-922.
Rigaut G., Shevchenko A., Rutz B., Wilm M., Mann M., Seraphin B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17:** 1030-1032.
Roberg K.J., Bickel S., Rowley N., Kaiser C.A. (1997). Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. *Genetics* **147:** 1569-1584.
Robertson L.S., Fink G.R. (1998). The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proc Natl Acad Sci U S A* **95:** 13783-13787.
Rock F.L., Mao W., Yaremchuk A., Tukalo M., Crepin T., Zhou H., Zhang Y.K., Hernandez V., Akama T., Baker S.J., Plattner J.J., Shapiro L., Martinis S.A., Benkovic S.J., Cusack S., Alley M.R.K. (2007). An Antifungal Agent Inhibits an Aminoacyl-tRNA Synthetase by Trapping tRNA in the Editing Site. *Science* **316:** 1759-1761.
Rodova M., Ankilova V., Safro M.G. (1999). Human phenylalanyl-tRNA synthetase: cloning, characterization of the deduced amino acid sequences in terms of the structural domains and

coordinately regulated expression of the alpha and beta subunits in chronic myeloid leukemia cells. *Biochem Biophys Res Commun* **255:** 765-773.
Rolfes R.J., Hinnebusch A.G. (1993). Translation of the yeast transcriptional activator GCN4 is stimulated by purine limitation: implications for activation of the protein kinase GCN2. *Mol Cell Biol* **13:** 5099-5111.
Romano P.R., Garcia-Barrio M.T., Zhang X., Wang Q., Taylor D.R., Zhang F., Herring C., Mathews M.B., Qin J., Hinnebusch A.G. (1998). Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2alpha kinases PKR and GCN2. *Mol Cell Biol* **18:** 2282-2297.
Rose M.D., Winston F.M., Hieter P. (1990). *Methods in yeast genetics: a laboratory course manual*. Cold Spring Harbor Laboratory Press.
Rossmann M.G., Moras D., Olsen K.W. (1974). Chemical and biological evolution of nucleotide-binding protein. *Nature* **250:** 194-199.
Roth A.F., Wan J., Bailey A.O., Sun B., Kuchar J.A., Green W.N., Phinney B.S., Yates J.R., 3rd, Davis N.G. (2006). Global analysis of protein palmitoylation in yeast. *Cell* **125:** 1003-1013.
Rouget P., Chapeville F. (1968). Reactions sequence of leucine activation catalysed by leucyl-RNA synthetase. 2. Formation of complexes between the enzyme and substrates. *Eur J Biochem* **4:** 310-314.
Rould M.A., Perona J.J., Soll D., Steitz T.A. (1989). Structure of E. coli glutaminyl-tRNA synthetase complexed with tRNA(Gln) and ATP at 2.8 A resolution. *Science* **246:** 1135-1142.
Rudoni S., Colombo S., Coccetti P., Martegani E. (2001). Role of guanine nucleotides in the regulation of the Ras/cAMP pathway in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **1538:** 181-189.
Rudra D., Mallick J., Zhao Y., Warner J.R. (2007). Potential interface between ribosomal protein production and pre-rRNA processing. *Mol Cell Biol* **27:** 4815-4824.
Ruff M., Krishnaswamy S., Boeglin M., Poterszman A., Mitschler A., Podjarny A., Rees B., Thierry J.C., Moras D. (1991). Class II aminoacyl transfer RNA synthetases: crystal structure of yeast aspartyl-tRNA synthetase complexed with tRNA(Asp). *Science* **252:** 1682-1689.
Russnak R., Konzcal D., McIntire S.L. (2001). A Family of Yeast Proteins Mediating Bidirectional Vacuolar Amino Acid Transport. *Journal of Biological Chemistry* **276:** 23849-23857.
Saier M.H., Jr. (2000). Families of transmembrane transporters selective for amino acids and their derivatives. *Microbiology* **146 ( Pt 8):** 1775-1795.
Sakai Y., Koller A., Rangell L.K., Keller G.A., Subramani S. (1998). Peroxisome degradation by microautophagy in Pichia pastoris: identification of specific steps and morphological intermediates. *J Cell Biol* **141:** 625-636.
Sambrook J. (2001). *Molecular cloning : a laboratory manual / Joseph Sambrook, David W. Russell*. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. :.
Sampath P., Mazumder B., Seshadri V., Gerber C.A., Chavatte L., Kinter M., Ting S.M., Dignam J.D., Kim S., Driscoll D.M., Fox P.L. (2004). Noncanonical function of glutamyl-prolyl-tRNA synthetase: gene-specific silencing of translation. *Cell* **119:** 195-208.
Sancak Y., Thoreen C.C., Peterson T.R., Lindquist R.A., Kang S.A., Spooner E., Carr S.A., Sabatini D.M. (2007). PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. *Molecular Cell* **25:** 903-915.
Sancak Y., Peterson T.R., Shaul Y.D., Lindquist R.A., Thoreen C.C., Bar-Peled L., Sabatini D.M. (2008). The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. *Science* **320:** 1496-1501.
Sancak Y., Bar-Peled L., Zoncu R., Markhard A.L., Nada S., Sabatini D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141:** 290-303.
Sankaranarayanan R., Moras D. (2001). The fidelity of the translation of the genetic code. *Acta Biochim Pol* **48:** 323-335.
Sarbassov D.D., Ali S.M., Kim D.H., Guertin D.A., Latek R.R., Erdjument-Bromage H., Tempst P., Sabatini D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* **14:** 1296-1302.

Sato T.K., Darsow T., Emr S.D. (1998). Vam7p, a SNAP-25-like molecule, and Vam3p, a syntaxin homolog, function together in yeast vacuolar protein trafficking. *Mol Cell Biol* **18:** 5308-5319.
Scandurro A.B., Weldon C.W., Figueroa Y.G., Alam J., Beckman B.S. (2001). Gene microarray analysis reveals a novel hypoxia signal transduction pathway in human hepatocellular carcinoma cells. *Int J Oncol* **19:** 129-135.
Schawalder S.B., Kabani M., Howald I., Choudhury U., Werner M., Shore D. (2004). Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Ifh1. *Nature* **432:** 1058-1061.
Scheper G.C., van der Klok T., van Andel R.J., van Berkel C.G., Sissler M., Smet J., Muravina T.I., Serkov S.V., Uziel G., Bugiani M., Schiffmann R., Krageloh-Mann I., Smeitink J.A., Florentz C., Van Coster R., Pronk J.C., van der Knaap M.S. (2007). Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. *Nat Genet* **39:** 534-539.
Scherens B., Feller A., Vierendeels F., Messenguy F., Dubois E. (2006). Identification of direct and indirect targets of the Gln3 and Gat1 activators by transcriptional profiling in response to nitrogen availability in the short and long term. *FEMS Yeast Res* **6:** 777-791.
Schmelzle T., Hall M.N. (2000). TOR, a Central Controller of Cell Growth. *Cell* **103:** 253-262.
Schmidt A., Beck T., Koller A., Kunz J., Hall M.N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. *EMBO J* **17:** 6924-6931.
Sehgal S.N. (2003). Sirolimus: its discovery, biological properties, and mechanism of action. *Transplant Proc* **35:** 7S-14S.
Sekito T., Fujiki Y., Ohsumi Y., Kakinuma Y. (2008). Novel families of vacuolar amino acid transporters. *IUBMB Life* **60:** 519-525.
Settembre C., Zoncu R., Medina D.L., Vetrini F., Erdin S., Huynh T., Ferron M., Karsenty G., Vellard M.C., Facchinetti V., Sabatini D.M., Ballabio A. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J* **31:** 1095-1108.
Shigemitsu K., Tsujishita Y., Hara K., Nanahoshi M., Avruch J., Yonezawa K. (1999a). Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. *J Biol Chem* **274:** 1058-1065.
Shigemitsu K., Tsujishita Y., Miyake H., Hidayat S., Tanaka N., Hara K., Yonezawa K. (1999b). Structural requirement of leucine for activation of p70 S6 kinase. *FEBS Lett* **447:** 303-306.
Shimazu M., Sekito T., Akiyama K., Ohsumi Y., Kakinuma Y. (2005). A family of basic amino acid transporters of the vacuolar membrane from Saccharomyces cerevisiae. *J Biol Chem* **280:** 4851-4857.
Shin C.S., Huh W.K. (2011). Bidirectional regulation between TORC1 and autophagy in Saccharomyces cerevisiae. *Autophagy* **7**.
Shin S.H., Kim H.S., Jung S.H., Xu H.D., Jeong Y.B., Chung Y.J. (2008). Implication of leucyl-tRNA synthetase 1 (LARS1) over-expression in growth and migration of lung cancer cells detected by siRNA targeted knock-down analysis. *Exp Mol Med* **40:** 229-236.
Smith D.B., Johnson K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene* **67:** 31-40.
Soulard A.a.C.A., Moes S., Schütz F., Jenö P., Hall M.N. (2010). The rapamycin-sensitive phosphoproteome reveals that TOR controls PKA toward some but not all substrates. *Molecular Biology of the Cell*.
Stanbrough M., Rowen D.W., Magasanik B. (1995). Role of the GATA factors Gln3p and Nil1p of Saccharomyces cerevisiae in the expression of nitrogen-regulated genes. *Proc Natl Acad Sci U S A* **92:** 9450-9454.
Stephan J.S., Yeh Y.Y., Ramachandran V., Deminoff S.J., Herman P.K. (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proc Natl Acad Sci U S A* **106:** 17049-17054.
Stern M., Jensen R., Herskowitz I. (1984). Five SWI genes are required for expression of the HO gene in yeast. *J Mol Biol* **178:** 853-868.
Stevens T.H., Forgac M. (1997). Structure, function and regulation of the vacuolar (H+)-ATPase. *Annu Rev Cell Dev Biol* **13:** 779-808.
Storey B.T., Fugere C., Lesieur-Brooks A., Vaslet C., Thompson N.L. (2005). Adenoviral modulation of the tumor-associated system L amino acid transporter, LAT1, alters amino acid transport, cell

growth and 4F2/CD98 expressionwith cell-type specific effects in cultured hepatic cells. *Int J Cancer* **117:** 387-397.
Sturgill T.W., Cohen A., Diefenbacher M., Trautwein M., Martin D.E., Hall M.N. (2008). TOR1 and TOR2 Have Distinct Locations in Live Cells. *Eukaryotic Cell* **7:** 1819-1830.
Talarek N., Cameroni E., Jaquenoud M., Luo X., Bontron S., Lippman S., Devgan G., Snyder M., Broach J.R., De Virgilio C. (2010). Initiation of the TORC1-regulated G0 program requires Igo1/2, which license specific mRNAs to evade degradation via the 5'-3' mRNA decay pathway. *Mol Cell* **38:** 345-355.
Tate J.J., Cooper T.G. (2003). Tor1/2 regulation of retrograde gene expression in Saccharomyces cerevisiae derives indirectly as a consequence of alterations in ammonia metabolism. *J Biol Chem* **278:** 36924-36933.
Teter S.A., Eggerton K.P., Scott S.V., Kim J., Fischer A.M., Klionsky D.J. (2001). Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J Biol Chem* **276:** 2083-2087.
Thevelein J.M., de Winde J.H. (1999). Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. *Mol Microbiol* **33:** 904-918.
Thomas D., Surdin-Kerjan Y. (1997). Metabolism of sulfur amino acids in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* **61:** 503-532.
Todaka Y., Wang Y., Tashiro K., Nakashima N., Nishimoto T., Sekiguchi T. (2005). Association of the GTP-binding protein Gtr1p with Rpc19p, a shared subunit of RNA polymerase I and III in yeast Saccharomyces cerevisiae. *Genetics* **170:** 1515-1524.
Tukalo M., Yaremchuk A., Fukunaga R., Yokoyama S., Cusack S. (2005). The crystal structure of leucyl-tRNA synthetase complexed with tRNALeu in the post-transfer-editing conformation. *Nat Struct Mol Biol* **12:** 923-930.
Uemura T., Tomonari Y., Kashiwagi K., Igarashi K. (2004). Uptake of GABA and putrescine by UGA4 on the vacuolar membrane in Saccharomyces cerevisiae. *Biochem Biophys Res Commun* **315:** 1082-1087.
Uno I., Matsumoto K., Adachi K., Ishikawa T. (1983). Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. *J Biol Chem* **258:** 10867-10872.
Upadhya R., Lee J., Willis I.M. (2002). Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol Cell* **10:** 1489-1494.
Urano J., Tabancay A.P., Yang W., Tamanoi F. (2000). The Saccharomyces cerevisiae Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J Biol Chem* **275:** 11198-11206.
Urban J., Soulard A., Huber A., Lippman S., Mukhopadhyay D., Deloche O., Wanke V., Anrather D., Ammerer G., Riezman H., Broach J.R., De Virgilio C., Hall M.N., Loewith R. (2007). Sch9 Is a Major Target of TORC1 in Saccharomyces cerevisiae. *Molecular Cell* **26:** 663-674.
Valbuena N., Guan K.L., Moreno S. (2012). The Vam6-Gtr1/Gtr2 pathway activates TORC1 in response to amino acids in fission yeast. *J Cell Sci*.
Van Hoof C., Martens E., Longin S., Jordens J., Stevens I., Janssens V., Goris J. (2005). Specific interactions of PP2A and PP2A-like phosphatases with the yeast PTPA homologues, Ypa1 and Ypa2. *Biochem J* **386:** 93-102.
Van Zeebroeck G., Bonini B.M., Versele M., Thevelein J.M. (2009). Transport and signaling via the amino acid binding site of the yeast Gap1 amino acid transceptor. *Nat Chem Biol* **5:** 45-52.
Verrey F., Closs E.I., Wagner C.A., Palacin M., Endou H., Kanai Y. (2004). CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch* **447:** 532-542.
Wade J.T., Hall D.B., Struhl K. (2004). The transcription factor Ifh1 is a key regulator of yeast ribosomal protein genes. *Nature* **432:** 1054-1058.
Wakasugi K., Schimmel P. (1999). Two distinct cytokines released from a human aminoacyl-tRNA synthetase. *Science* **284:** 147-151.
Wang C.W., Klionsky D.J. (2003). The molecular mechanism of autophagy. *Mol Med* **9:** 65-76.
Wang L., Harris T.E., Roth R.A., Lawrence J.C., Jr. (2007). PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J Biol Chem* **282:** 20036-20044.

Wang X., Fonseca B.D., Tang H., Liu R., Elia A., Clemens M.J., Bommer U.A., Proud C.G. (2008). Re-evaluating the Roles of Proposed Modulators of Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling. *Journal of Biological Chemistry* **283:** 30482-30492.
Wanke V., Pedruzzi I., Cameroni E., Dubouloz F., De Virgilio C. (2005). Regulation of G0 entry by the Pho80-Pho85 cyclin-CDK complex. *EMBO J* **24:** 4271-4278.
Wanke V., Cameroni E., Uotila A., Piccolis M., Urban J., Loewith R., De Virgilio C. (2008). Caffeine extends yeast lifespan by targeting TORC1. *Mol Microbiol* **69:** 277-285.
Warner J.R. (1999). The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* **24:** 437-440.
Wasenius V.M., Hemmer S., Kettunen E., Knuutila S., Franssila K., Joensuu H. (2003). Hepatocyte growth factor receptor, matrix metalloproteinase-11, tissue inhibitor of metalloproteinase-1, and fibronectin are up-regulated in papillary thyroid carcinoma: a cDNA and tissue microarray study. *Clin Cancer Res* **9:** 68-75.
Watson T.G. (1976). Amino-acid pool composition of Saccharomyces cerevisiae as a function of growth rate and amino-acid nitrogen source. *J Gen Microbiol* **96:** 263-268.
Webb J.L., Ravikumar B., Atkins J., Skepper J.N., Rubinsztein D.C. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* **278:** 25009-25013.
Wedaman K.P., Reinke A., Anderson S., Yates J., 3rd, McCaffery J.M., Powers T. (2003). Tor kinases are in distinct membrane-associated protein complexes in Saccharomyces cerevisiae. *Mol Biol Cell* **14:** 1204-1220.
Wei M., Fabrizio P., Hu J., Ge H., Cheng C., Li L., Longo V.D. (2008). Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. *PLoS Genet* **4:** e13.
Wei Y., Tsang C.K., Zheng X.F.S. (2009). Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1. *The EMBO Journal* **28:** 2220-2230.
Wek R.C., Jackson B.M., Hinnebusch A.G. (1989). Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability. *Proc Natl Acad Sci U S A* **86:** 4579-4583.
Williams A.M., Martinis S.A. (2006). Mutational unmasking of a tRNA-dependent pathway for preventing genetic code ambiguity. *Proc Natl Acad Sci U S A* **103:** 3586-3591.
Wilson B.S., Deanin G.G., Standefer J.C., Vanderjagt D., Oliver J.M. (1989). Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. *J Immunol* **143:** 259-265.
Wilson L.K., Benton B.M., Zhou S., Thorner J., Martin G.S. (1995). The yeast immunophilin Fpr3 is a physiological substrate of the tyrosine-specific phosphoprotein phosphatase Ptp1. *J Biol Chem* **270:** 25185-25193.
Wu B., Ottow K., Poulsen P., Gaber R.F., Albers E., Kielland-Brandt M.C. (2006). Competitive intra- and extracellular nutrient sensing by the transporter homologue Ssy1p. *J Cell Biol* **173:** 327-331.
Wullschleger S., Loewith R., Oppliger W., Hall M.N. (2005). Molecular organization of target of rapamycin complex 2. *J Biol Chem* **280:** 30697-30704.
Xu G., Kwon G., Marshall C.A., Lin T.-A., Lawrence J.C., McDaniel M.L. (1998). Branched-chain Amino Acids Are Essential in the Regulation of PHAS-I and p70 S6 Kinase by Pancreatic Œ≤-Cells. *Journal of Biological Chemistry* **273:** 28178-28184.
Xue Y., Batlle M., Hirsch J.P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. *EMBO J* **17:** 1996-2007.
Yalowitz J.A., Jayaram H.N. (2000). Molecular targets of guanine nucleotides in differentiation, proliferation and apoptosis. *Anticancer Res* **20:** 2329-2338.
Yan G., Shen X., Jiang Y. (2006). Rapamycin activates Tap42-associated phosphatases by abrogating their association with Tor complex 1. *EMBO J* **25:** 3546-3555.
Yang X.L., Schimmel P., Ewalt K.L. (2004). Relationship of two human tRNA synthetases used in cell signaling. *Trends Biochem Sci* **29:** 250-256.
Yang Z., Huang J., Geng J., Nair U., Klionsky D.J. (2006). Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. *Mol Biol Cell* **17:** 5094-5104.

Yang Z., Klionsky D.J. (2007). Permeases recycle amino acids resulting from autophagy. *Autophagy* **3:** 149-150.
Yannay-Cohen N., Carmi-Levy I., Kay G., Yang C.M., Han J.M., Kemeny D.M., Kim S., Nechushtan H., Razin E. (2009). LysRS serves as a key signaling molecule in the immune response by regulating gene expression. *Mol Cell* **34:** 603-611.
Yao K., Yin Y.L., Chu W., Liu Z., Deng D., Li T., Huang R., Zhang J., Tan B., Wang W., Wu G. (2008a). Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. *J Nutr* **138:** 867-872.
Yao P., Zhou X.L., He R., Xue M.Q., Zheng Y.G., Wang Y.F., Wang E.D. (2008b). Unique Residues Crucial for Optimal Editing in Yeast Cytoplasmic Leucyl-tRNA Synthetase Are Revealed by Using a Novel Knockout Yeast Strain. *Journal of Biological Chemistry* **283:** 22591-22600.
Yip C.K., Murata K., Walz T., Sabatini D.M., Kang S.A. (2010). Structure of the Human mTOR Complex I and Its Implications for Rapamycin Inhibition. *Molecular Cell* **38:** 768-774.
Yorimitsu T., Zaman S., Broach J.R., Klionsky D.J. (2007). Protein kinase A and Sch9 cooperatively regulate induction of autophagy in Saccharomyces cerevisiae. *Mol Biol Cell* **18:** 4180-4189.
Zahringer H., Thevelein J.M., Nwaka S. (2000). Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. *Mol Microbiol* **35:** 397-406.
Zaman S., Lippman S.I., Zhao X., Broach J.R. (2008). HowSaccharomycesResponds to Nutrients. *Annual Review of Genetics* **42:** 27-81.
Zaragoza D., Ghavidel A., Heitman J., Schultz M.C. (1998). Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol Cell Biol* **18:** 4463-4470.
Zargari A., Boban M., Heessen S., Andreasson C., Thyberg J., Ljungdahl P.O. (2007). Inner nuclear membrane proteins Asi1, Asi2, and Asi3 function in concert to maintain the latent properties of transcription factors Stp1 and Stp2. *J Biol Chem* **282:** 594-605.
Zeller C.E., Parnell S.C., Dohlman H.G. (2007). The RACK1 ortholog Asc1 functions as a G-protein beta subunit coupled to glucose responsiveness in yeast. *J Biol Chem* **282:** 25168-25176.
Zeng X., Wang F., Fan X., Yang W., Zhou B., Li P., Yin Y., Wu G., Wang J. (2008). Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. *J Nutr* **138:** 1421-1425.
Zervos A.S., Gyuris J., Brent R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* **72:** 223-232.
Zheng Y., Jiang Y. (2005). The yeast phosphotyrosyl phosphatase activator is part of the Tap42-phosphatase complexes. *Mol Biol Cell* **16:** 2119-2127.
Zoncu R., Bar-Peled L., Efeyan A., Wang S., Sancak Y., Sabatini D.M. (2011). mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H+-ATPase. *Science* **334:** 678-683.
Zurita-Martinez S.A., Puria R., Pan X., Boeke J.D., Cardenas M.E. (2007). Efficient Tor signaling requires a functional class C Vps protein complex in Saccharomyces cerevisiae. *Genetics* **176:** 2139-2150.

# - Appendix -

Publications:

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Molecular Cell
Short Article

# Leucyl-tRNA Synthetase Controls TORC1 via the EGO Complex

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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 misacylated $tRNA^{Leu}$ 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

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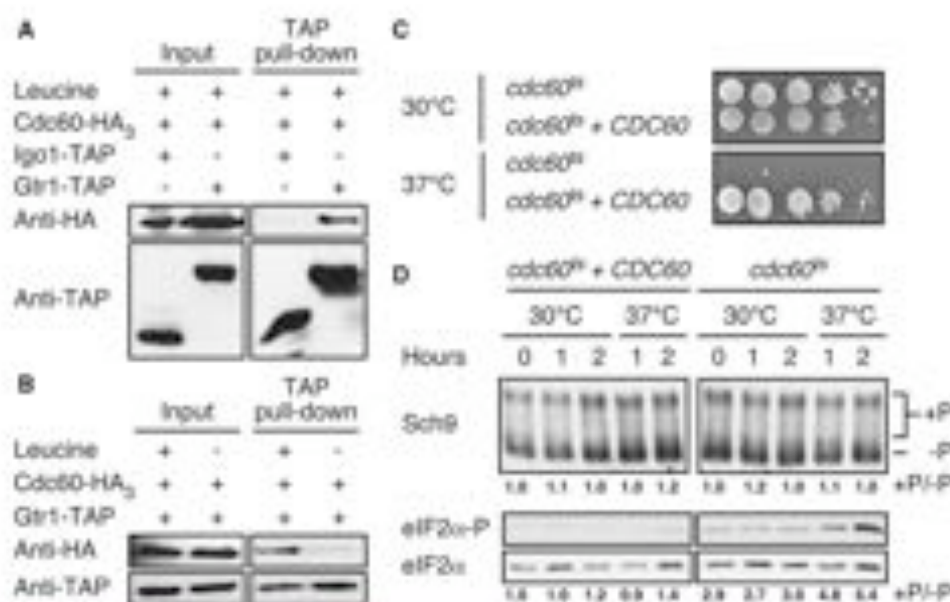


**Figure 1. LeuRS Cdc60 Physically Interacts with the TORC1 Regulator Gtr1 in a Leucine-Dependent Manner, but Cdc60-Mediated tRNA$^{Leu}$ Aminoacylation Is Not Required for Normal TORC1 Activity**

(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

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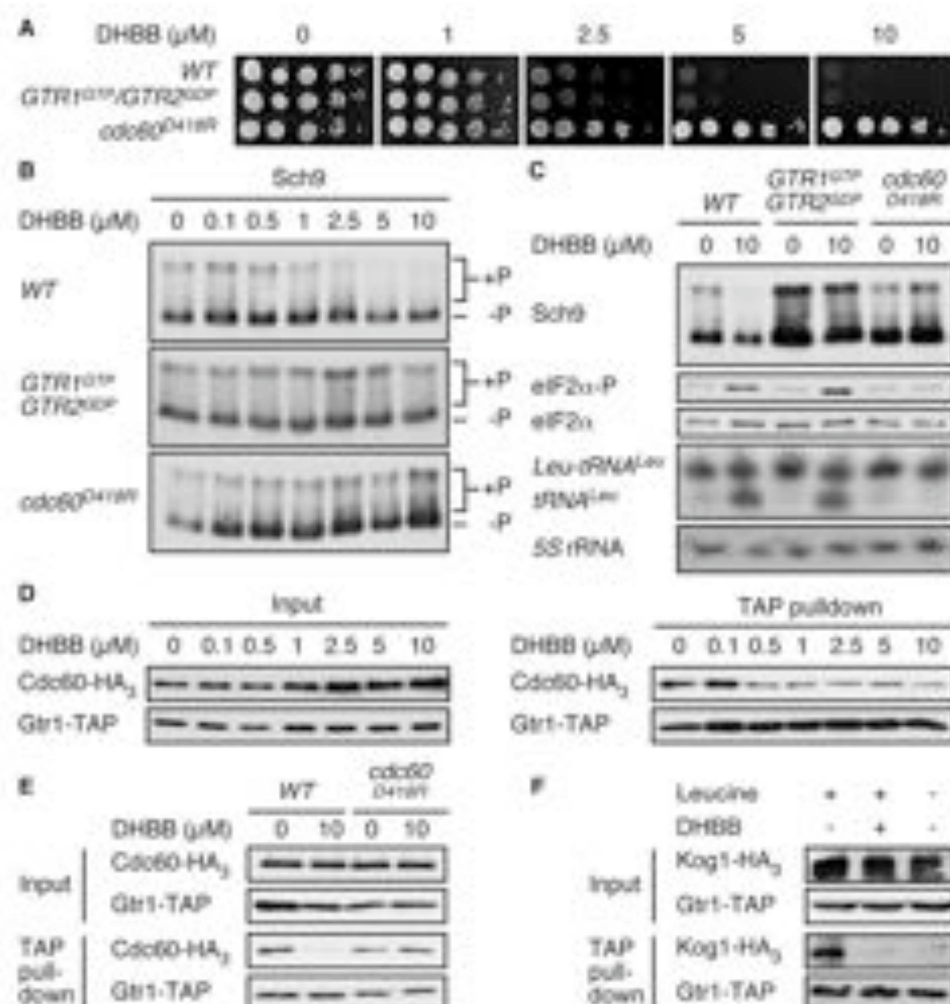


**Figure 2. DHBB-Mediated Trapping of tRNA$^{Leu}$ within the LeuRS Editing Site Downregulates EGOC-TORC1 Signaling**

(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. DHBB 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

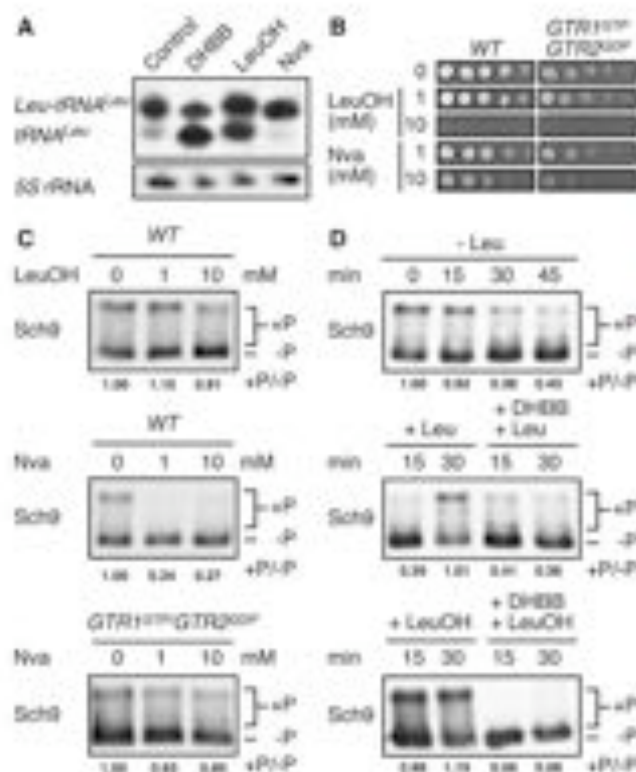


**Figure 3. LeuRS Inhibitors Leucinol (LeuOH) and Norvaline (Nva) Dampen and Stimulate LeuRS Editing, Respectively, and Oppositely Affect EGOC-TORC1 Signaling**

(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 7.6 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$^{Cdc60-S414F}$, 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 Regulator-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

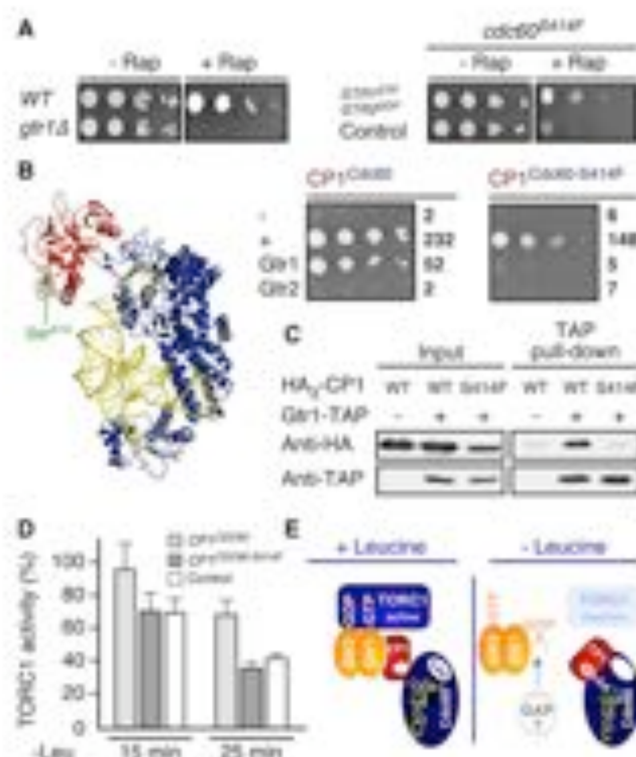


**Figure 4. Mutation of Ser[414] to Phe within the CP1 Domain of Cdc60 Disrupts Its Interaction with Gtr1**

(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$^{Cdc60-S414F}$, 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$^{Cdc60-S414F}$-Cub. pOL2-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$^{CP1-S414F}$ (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$^{Cdc60-S414F}$, partially protects TORC1 (quantified as in Figure 1C) 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 (Taliansk et al., 2010). Co-IP 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 PPi) 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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## REFERENCES

Alexis, S.F., and Silva, V. (2006). Small molecules, big players in the evolution of protein synthesis. ACS Chem. Biol. 1, 285–297.

Avruch, J., Long, X., Ortiz-Vega, S., Rapley, J., Papageorgiou, A., and Dai, N. (2009). Amino acid regulation of TOR complex 1. Am. J. Physiol. Endocrinol. Metab. 296, E592–E602.

Binda, M., Péli-Gulli, M.P., Bonfils, G., Panchaud, N., Urban, J., Sturgill, T.W., Loewith, R., and De Virgilio, C. (2009). The Vam6 GEF controls TORC1 by activating the EGO complex. Mol. Cell 35, 563–573.

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Chen, X., Ma, J.J., Tan, M., Yao, P., Hu, Q.H., Eriani, G., and Wang, E.D. (2011). Modular pathways for editing non-cognate amino acids by human cytoplasmic leucyl-tRNA synthetase. Nucleic Acids Res. 39, 235–247.

Christie, G.R., Hajduch, E., Hundal, H.S., Proud, C.G., and Taylor, P.M. (2002). Intracellular sensing of amino acids in Xenopus laevis oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. J. Biol. Chem. 277, 9952–9957.

Dubouloz, F., Deloche, O., Wanke, V., Cameroni, E., and De Virgilio, C. (2005). The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol. Cell 19, 15–26.

Echols, N., Harrison, P., Balasubramanian, S., Luscombe, N.M., Bertone, P., Zhang, Z., and Gerstein, M. (2002). Comprehensive analysis of amino acid and nucleotide composition in eukaryotic genomes, comparing genes and pseudogenes. Nucleic Acids Res. 30, 2515–2523.

Forsburg, S.L. (2001). The art and design of genetic screens: yeast. Nat. Rev. Genet. 2, 659–668.

Gao, M., and Kaiser, C.A. (2006). A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. Nat. Cell Biol. 8, 657–667.

Gelperin, D.M., White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., et al. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev. 19, 2816–2826.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature 425, 737–741.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer Cell 12, 9–22.

Hinnebusch, A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. Annu. Rev. Microbiol. 59, 407–450.

Hohmann, S., and Thevelein, J.M. (1992). The cell division cycle gene CDC60 encodes cytosolic leucyl-tRNA synthetase in Saccharomyces cerevisiae. Gene 120, 43–49.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nat. Cell Biol. 10, 935–945.

Kogan, K., Spear, E.D., Kaiser, C.A., and Fass, D. (2010). Structural conservation of components in the amino acid sensing branch of the TOR pathway in yeast and mammals. J. Mol. Biol. 402, 388–398.

Köhrer, C., and RajBhandary, U.L. (2008). The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. Methods 44, 129–138.

Ling, J., Reynolds, N., and Ibba, M. (2009). Aminoacyl-tRNA synthesis and translational quality control. Annu. Rev. Microbiol. 63, 61–78.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell 10, 457–468.

Rock, F.L., Mao, W., Yaremchuk, A., Tukalo, M., Crépin, T., Zhou, H., Zhang, Y.K., Hernandez, V., Akama, T., Baker, S.J., et al. (2007). An antifungal agent inhibits an aminoacyl-tRNA synthetase by trapping tRNA in the editing site. Science 316, 1759–1761.

Rouget, P., and Chapeville, F. (1968). Reaction sequence of leucine activation catalysed by leucyl-RNA synthetase. 1. Kinetic studies. Eur. J. Biochem. 4, 305–309.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 290–303.

Shin, S.H., Kim, H.S., Jung, S.H., Xu, H.D., Jeong, Y.B., and Chung, Y.J. (2008). Implication of leucyl-tRNA synthetase 1 (LARS1) over-expression in growth and migration of lung cancer cells detected by siRNA targeted knock-down analysis. Exp. Mol. Med. 40, 229–236.

Talarek, N., Cameroni, E., Jaquenoud, M., Luo, X., Bontron, S., Lippman, S., Devgan, G., Snyder, M., Broach, J.R., and De Virgilio, C. (2010). Initiation of the TORC1-regulated $G_0$ program requires Igo1/2, which license specific mRNAs to evade degradation via the 5'-3' mRNA decay pathway. Mol. Cell 38, 345–355.

Tukalo, M., Yaremchuk, A., Fukunaga, R., Yokoyama, S., and Cusack, S. (2005). The crystal structure of leucyl-tRNA synthetase complexed with $tRNA^{Leu}$ in the post-transfer-editing conformation. Nat. Struct. Mol. Biol. 12, 923–930.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., et al. (2007). Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol. Cell 26, 663–674.

Wang, X., Fonseca, B.D., Tang, H., Liu, R., Elia, A., Clemens, M.J., Bommer, U.A., and Proud, C.G. (2008). Re-evaluating the roles of proposed modulators of mammalian target of rapamycin complex 1 (mTORC1) signaling. J. Biol. Chem. 283, 30482–30492.

Wanke, V., Cameroni, E., Uotila, A., Piccolis, M., Urban, J., Loewith, R., and De Virgilio, C. (2008). Caffeine extends yeast lifespan by targeting TORC1. Mol. Microbiol. 69, 277–285.

Yao, P., Zhou, X.L., He, R., Xue, M.Q., Zheng, Y.G., Wang, Y.F., and Wang, E.D. (2008). Unique residues crucial for optimal editing in yeast cytoplasmic Leucyl-tRNA synthetase are revealed by using a novel knockout yeast strain. J. Biol. Chem. 283, 22591–22600.

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Article

# The Vam6 GEF Controls TORC1 by Activating the EGO Complex

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## SUMMARY

**The target of rapamycin complex 1 (TORC1) is a central regulator of eukaryotic cell growth that is activated by a variety of hormones (e.g., insulin) and nutrients (e.g., amino acids) and is deregulated in various cancers. Here, we report that the yeast Rag GTPase homolog Gtr1, a component of the vacuolar-membrane-associated EGO complex (EGOC), interacts with and activates TORC1 in an amino-acid-sensitive manner. Expression of a constitutively active (GTP-bound) Gtr1$^{GTP}$, which interacted strongly with TORC1, rendered TORC1 partially resistant to leucine deprivation, whereas expression of a growth inhibitory, GDP-bound Gtr1$^{GDP}$, caused constitutively low TORC1 activity. We also show that the nucleotide-binding status of Gtr1 is regulated by the conserved guanine nucleotide exchange factor (GEF) Vam6. Thus, in addition to its regulatory role in homotypic vacuolar fusion and vacuole protein sorting within the HOPS complex, Vam6 also controls TORC1 function by activating the Gtr1 subunit of the EGO complex.**

## INTRODUCTION

The target of rapamycin complex 1 (TORC1) is a structurally and functionally conserved, essential multiprotein complex that positively regulates cell growth by promoting anabolic processes (e.g., protein synthesis) and inhibiting catabolic processes (e.g., autophagy) in response to diverse signals, including mitogenic growth factors, energy/ATP levels, and amino acids (De Virgilio and Loewith, 2006a; Wullschleger et al., 2006). The mechanisms that couple growth factors and energy levels to mammalian TORC1 (mTORC1) have been characterized in considerable detail and implicate the phosphatidylinositol 3-kinase (PI3K), Akt, TSC1/TSC2, and Rheb, a small guanosine triphosphate (GTP)-binding protein that directly binds to and stimulates mTORC1 (Sarbassov et al., 2005). The mechanisms through which amino acids signal to TORC1, in contrast, have largely remained elusive until very recently. Two complementary studies have reported that the conserved Rag GTPases act as upstream regulators of TORC1 and play important roles in coupling amino-acid-derived signals to TORC1 in both *Drosophila* and mammalian cells (Kim et al., 2008; Sancak et al., 2008). RagA and RagB are very similar to each other and orthologous to yeast Gtr1, whereas RagC and RagD are similar and orthologous to yeast Gtr2 (Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). Rag and Gtr proteins function in heterodimeric complexes, which contain one Gtr1-like GTPase and one Gtr2-like GTPase (Nakashima et al., 1999; Sekiguchi et al., 2001). Importantly, amino acid signals are thought to impinge on GTP loading of RagA/B, but it is not known which factors regulate the GTP loading of these GTPases.

We previously reported that the EGO complex in yeast, consisting of Ego1/Meh1, Ego3/Slm4, Gtr2, and Gtr1, which was identified subsequently (Gao and Kaiser, 2006; Gao et al., 2005), may function upstream of TORC1 to mediate amino acid signaling (De Virgilio and Loewith, 2006a, 2006b; Dubouloz et al., 2005). In this study, we provide evidence that the EGO complex indeed functions directly upstream of TORC1. TORC1 activity is dictated by the nucleotide-bound state of Gtr1, and this is dependent on the presence of Tco89, a nonessential component of TORC1. Furthermore, we demonstrate that Vam6 colocalizes with the EGO complex/TORC1 at the limiting membrane of the vacuole and functions as a guanine nucleotide exchange factor (GEF) for Gtr1. Thus, in addition to its regulatory role in homotypic vacuolar fusion and vacuole protein sorting as part of the HOPS complex, Vam6 also controls the activity of TORC1 by activating the Gtr1 subunit of the EGO complex.

## RESULTS AND DISCUSSION

### EGOC Acts Upstream of TORC1

Loss of EGO complex (Ego1, Ego3, Gtr1, and Gtr2) or TORC1 (Tco89) subunits results in an inability to restart growth following exposure to rapamycin (Figure 1A; Dubouloz et al., 2005). Similar to wild-type cells treated with rapamycin or the more recently described TORC1 inhibitor caffeine (Kuranda et al., 2006; Reinke et al., 2006; Wanke et al., 2008), these mutants also possess decreased TORC1 activity as assessed by monitoring phosphorylation of the TORC1 substrate Sch9 (Urban et al., 2007) (Figure 1A). Moreover, the potent increase in TORC1 activity observed in wild-type cells treated with cycloheximide, a translation elongation inhibitor that may indirectly boost the levels of free intracellular amino acids (Beugnet et al., 2003; Urban

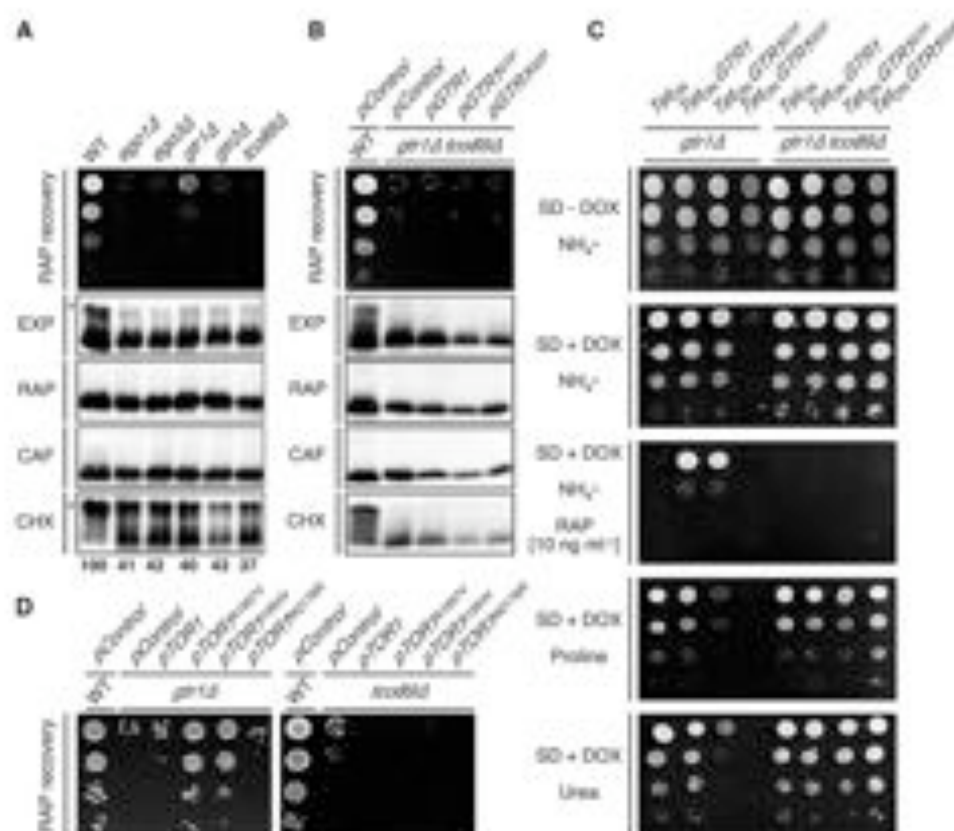


**Figure 1. EGOC Acts Upstream of TORC1**

(A) Loss of TORC1 (Tco89) or EGOC (Ego1, Ego3, Gtr1, and Gtr2) subunits causes a defect in recovery from rapamycin-induced growth arrest and decreases TORC1 activity. (Top) Wild-type and isogenic mutant cells were grown exponentially in YPD, treated for 6 hr with rapamycin (200 ng $ml^{-1}$), and then washed twice and spotted as 10-fold serial dilutions on YPD plates. (Bottom) Indicated strains expressing a plasmid-based copy of $SCH9^{T570A}$-$HA_5$ were grown exponentially (in YPD + 0.2% glutamine; EXP) and treated for 30 min with rapamycin (RAP; 200 ng $ml^{-1}$), caffeine (CAF; 20 mM), or cycloheximide (CHX; 25 $\mu g$ $ml^{-1}$). Protein extracts were prepared and subsequently treated with NTCB to cleave polypeptide chains at cysteine residues (Urban et al., 2007). Aliquots were then analyzed by SDS-PAGE, and immunoblots were probed with anti-HA antibodies (only the migration pattern of the C terminus of Sch9 is shown). The extent of Sch9 phosphorylation (and, by proxy, TORC1 activity) was determined by quantifying the signal of the slowest migrating (most highly phosphorylated) species of Sch9 (*) and dividing this by the total signal observed for all species. For comparison, this ratio was set to 100% for wild-type cells and calculated correspondingly (in percent) for the indicated mutants (numbers below lowest chart; standard deviations were below 5% in each case).

(B) $Gtr1^{GTP}$ suppresses neither the defects in recovery following rapamycin treatment nor the intrinsically low TORC1 activity in *tco89Δ* cells. Wild-type and double *gtr1Δ tco89Δ* mutant strains were transformed with an empty vector or vectors that express *GTR1*, $GTR1^{GTP}$, or $GTR1^{GDP}$ from the endogenous promoter and were assayed as in (A).

(C) Growth inhibition following overproduction of $Gtr1^{GDP}$ and growth inhibition on nitrogen-poor (proline- and urea-containing) media following overproduction of $Gtr1^{GTP}$ depend on the presence of Tco89. Single *gtr1Δ* and double *gtr1Δ tco89Δ* mutant strains were transformed with an empty vector or vectors that express *GTR1*, $GTR1^{GTP}$, or $GTR1^{GDP}$ from the doxycycline-inducible $Tet_{ON}$ promoter, were grown to exponential phase in doxycycline-containing media, and were spotted on plates containing, or not, doxycyline (DOX; 5 $\mu g$ $ml^{-1}$) and rapamycin (RAP; 10 ng $ml^{-1}$) and either 75 mM $NH_4^+$ (top three panels), 10 mM proline, or 10 mM urea as nitrogen source as indicated.

(D) Hyperactive $TOR1^{A1957V}$ and $TOR1^{I1954V}$ alleles, unlike wild-type or the hypoactive $TOR1^{W2176R}$ allele, suppress the defect in recovery following rapamycin treatment in *gtr1Δ*, but not *tco89Δ* cells. Wild-type and single *gtr1Δ* and *tco89Δ* mutant strains were transformed with an empty vector or vectors that express *TOR1*, $TOR1^{A1957V}$, $TOR1^{I1954V}$, or $TOR1^{W2176R}$ from the endogenous promoter and were assayed as in (A).

et al., 2007), was partially dependent on the presence of Ego1, Ego3, Gtr1, or Gtr2, further supporting the idea that the EGO complex may function upstream of TORC1 (Figure 1A). Observations from several genetic experiments are in line with this interpretation. First, expression of $Gtr1^{GTP}$ or $Gtr1^{GDP}$ alleles, which are predicted to be restricted to either a GTP- or GDP-bound conformation (Gao and Kaiser, 2006; Nakashima et al., 1999), did not alter the intrinsically low TORC1 activity or the inability to recover following rapamycin treatment of *tco89Δ* cells (Figure 1B). Second, overexpression of $Gtr1^{GDP}$ reduced growth rate on rich media, whereas overexpression of $Gtr1^{GTP}$ reduced growth rate on media containing poor nitrogen sources (proline and urea), and these phenotypes were suppressed in *tco89Δ* cells (Figure 1C). Third, hyperactive $TOR1^{A1957V}$ and $TOR1^{I1954V}$ alleles (Reinke et al., 2006), unlike wild-type *TOR1* or the hypoactive $TOR1^{W2176R}$ allele, fully suppressed the defect in recovery following rapamycin treatment in *gtr1Δ* cells, but not in *tco89Δ* cells (Figure 1D). Lastly, TORC1 inhibition results in the activation

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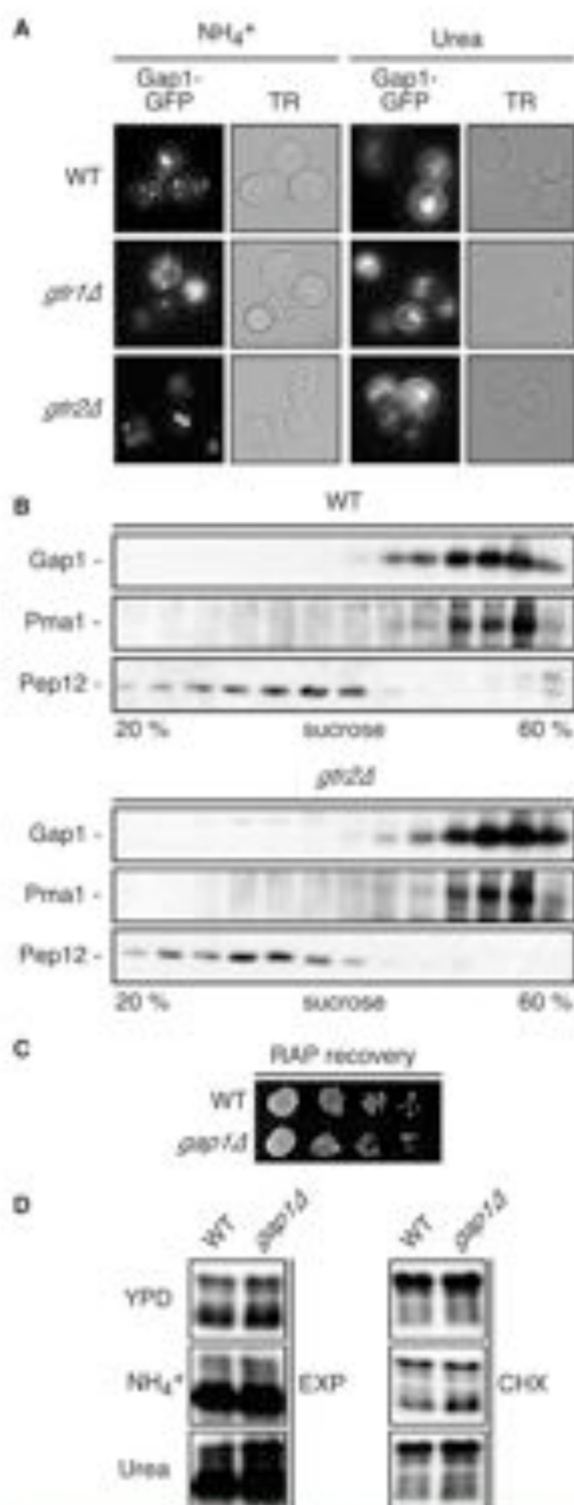


**Figure 2. EGOC Does Not Control TORC1 Indirectly via Gap1 Sorting**

(A) EGO complex subunits (i.e., Gtr1 or Gtr2) are not required for sorting Gap1 to the plasma membrane. Prototrophic wild-type, *gtr1Δ*, and *gtr2Δ* strains expressing a plasmid-encoded Gap1-GFP under the control of the galactose-inducible *GAL1* promoter were pregrown on SD medium (containing 75 mM $NH_4^+$ as nitrogen source and 2% raffinose/0.1% sucrose as carbon source). Cells were then incubated for 2 hr in the presence of 2% galactose (to induce Gap1-GFP expression) and analyzed by fluorescence microscopy either directly ($NH_4^+$) or following an additional 2 hr incubation on SD media containing 10 mM urea as nitrogen source (urea) and 2% glucose (to repress Gap1-GFP de novo synthesis). As reported previously, overproduction of Gap1-GFP from the *GAL1* promoter causes some accumulation of Gap1-GFP in the vacuole (De Craene et al., 2001). TR, transmission.

(B) Prototrophic wild-type and *gtr2Δ* cells growing on SD medium containing 10 mM urea as nitrogen source were lysed and fractionated by equilibrium density centrifugation on continuous 20%–60% sucrose gradients (Kaiser et al., 2002). The fractions were assayed for the presence of Gap1, the plasma membrane marker protein Pma1, or the endosomal marker protein Pep12 using specific antibodies that recognize the corresponding proteins.

(C) Gap1 is not required for recovery of cells following rapamycin treatment. Prototrophic wild-type and *gap1Δ* cells were treated as in Figure 1A.

(D) Gap1 is neither required for normal TORC1 activity in exponentially growing cells (EXP) nor for TORC1 activation by cycloheximide (CHX). Prototrophic wild-type and *gap1Δ* cells were grown on YPD (+ 0.2% glutamine), or on SD media containing 75 mM $NH_4^+$ ($NH_4^+$) or 10 mM urea (urea) as nitrogen source and were assayed for TORC1 activity as in Figure 1A.

and nuclear localization of the transcription factor Gln3 (Beck and Hall, 1999), an event that is growth inhibitory for cells. Deletion of *GLN3* suppressed the defect in recovery from rapamycin treatment, but not the TORC1 activity defect of EGO complex mutants (Figure S1 available online). Collectively, these observations support the idea that the EGO complex signals, both positively and negatively, to TORC1 and further suggest that this signal is mediated by the TORC1 component Tco89.

### EGOC Does Not Control TORC1 Indirectly via Gap1 Sorting

In a previous study (Gao and Kaiser, 2006), all four subunits of the EGO complex were shown to be required for intracellular sorting of the general amino acid permease Gap1, a process that is controlled by the quality of the external nitrogen source. Accordingly, in wild-type cells grown on a relatively poor nitrogen source such as urea, Gap1 is sorted to the plasma membrane (where it is active for transport), whereas in cells grown on rich nitrogen sources such as glutamate/glutamine or ammonium ($NH_4^+$), active Gap1 is internalized and directed to the vacuole for degradation (Springael and André, 1998), and newly synthesized Gap1 is directly sorted from the *trans*-Golgi to the vacuole (De Craene et al., 2001). Although we observed that loss of EGOC affected TORC1 activity even when cells were grown on rich nitrogen sources, i.e., under conditions in which Gap1 is transcriptionally repressed and not sorted to the plasma membrane (De Craene et al., 2001; Jauniaux and Grenson, 1990), we decided to address the possibility that the EGOC may indirectly affect TORC1 activity via a potential effect on Gap1 sorting. To our surprise, examination of the subcellular distribution of Gap1-GFP, which was transiently expressed from a galactose-inducible *GAL1* promoter (Nikko et al., 2003), revealed that sorting of Gap1 from exclusively intracellular compartments (in cells grown on $NH_4^+$-containing medium) to the plasma membrane (in cells shifted to urea-containing medium) occurred normally in both wild-type and *egoc* mutant cells (Figure 2A). Similarly, when assayed by subcellular fractionation analysis, loss of Gtr2, which was suggested to be particularly important for Gap1 sorting (Gao and Kaiser, 2006), had no impact on the cells' ability to

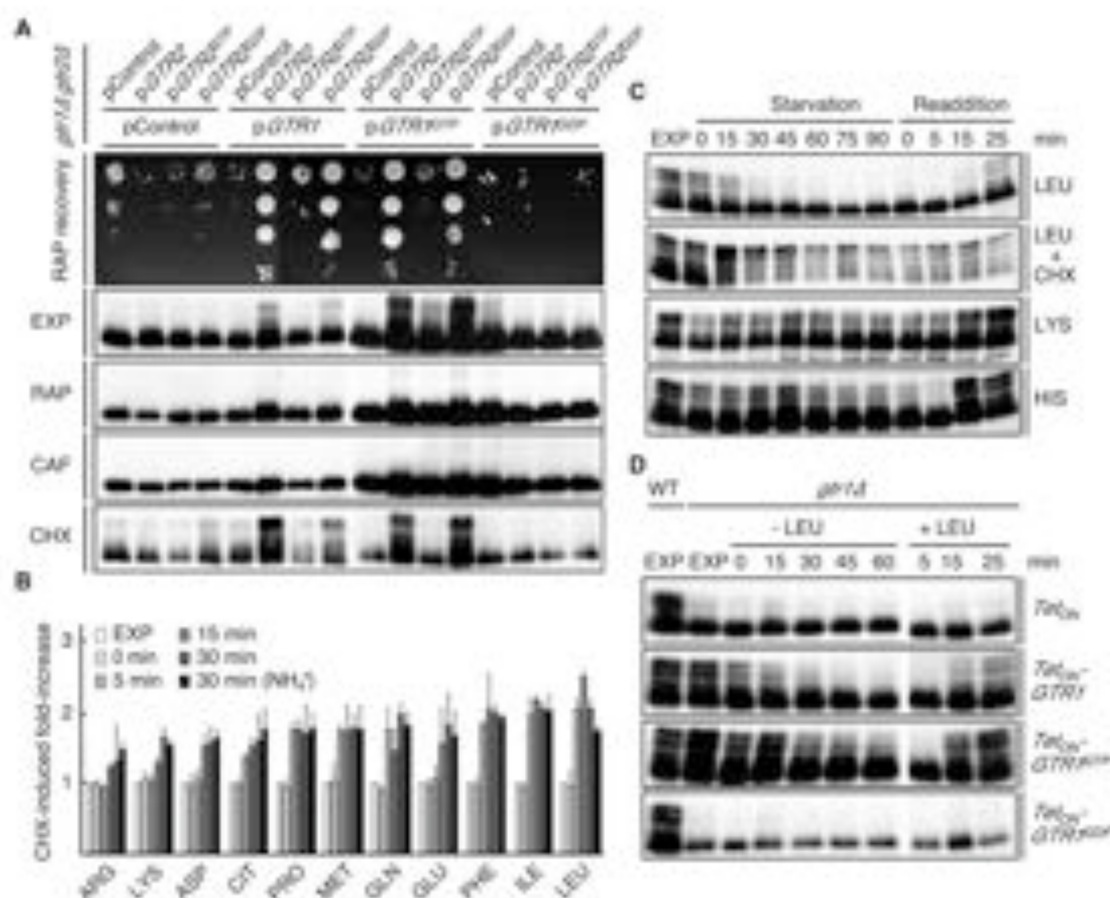


**Figure 3. Alternative Nucleotide-Bound States of Gtr1 and Gtr2 Are Required for Amino-Acid-Dependent Control of TORC1**

(A) The nucleotide-binding states of Gtr1 and Gtr2 control both the cell's ability to recover from rapamycin treatment and TORC1 activity. Rapamycin recovery and TORC1 activity assays (as in Figure 1A) were determined in a *gtr1Δ gtr2Δ* double-mutant strain carrying all combinations of plasmids between pGTR1, pGTR1$^{S20L}$, or pGTR1$^{Q65L}$ and pGTR2, pGTR2$^{S23L}$, or pGTR2$^{Q66L}$.

(B) Intracellular pools of free amino acids increase rapidly following CHX treatment. Intracellular, free amino acids were determined in prototrophic wild-type cells subjected to CHX (25 μg ml$^{-1}$) treatment for the times indicated and expressed as fold increase compared to the levels detected in exponentially growing (EXP) cells. Cells were grown on YPD (+ 0.2% glutamine) or (as indicated) on SD without amino acids ($NH_4^+$). Experiments were done in triplicate and expressed as mean + SD. As assessed by one-way analysis of variance (ANOVA) followed by posttest analysis, the CHX-induced increases are statistically significant for all amino acids (except for arginine) with P values that are < 0.05 for glutamate and methionine and < 0.001 for all other amino acids.

(C) TORC1 is reversibly inactivated in response to amino acid starvation. Leucine (*leu2Δ*, top two panels), lysine (*lys2Δ*), and histidine (*his3Δ*) auxotrophic wild-type strains were grown to exponential phase (EXP) in medium containing leucine (LEU), lysine (LYS), or histidine (HIS), respectively, and were then transferred to a medium lacking the corresponding amino acid. Leucine (2.8 mM), lysine (0.4 mM), and histidine (0.5 mM) were readded after 90 min of starvation. Samples were taken at the times indicated following the medium changes and were assayed as in Figure 1A. The leucine starvation experiment was also carried out in the presence of cycloheximide (25 μg ml$^{-1}$), which was added at time point 0 (before starvation; second panel from top).

(D) TORC1 is partially insensitive to leucine deprivation in cells expressing Gtr1$^{Q65L}$. Leucine auxotrophic wild-type and *gtr1Δ* cells carrying the empty vector or vectors that express GTR1, GTR1$^{S20L}$, or GTR1$^{Q65L}$ from the doxycycline-inducible $Tet_{ON}$ promoter were assayed for TORC1 activity (as in C).

sort Gap1 to the plasma membrane when grown on urea-containing medium (Figure 2B).

In trying to understand why our above results were so strikingly different from the previously reported observations, we noted that, in contrast to various wild-type strains, including Σ1278b (Grenson, 1983), KT1960 (see below), and the S288C-derived BY4741/2 (Brachmann et al., 1998) used here, the wild-type strain used by the Kaiser group appears to be defective for $NH_4^+$-inactivation of Gap1 (Gao and Kaiser, 2006). In this context, Roberg and colleagues have previously noted that this particular phenotype of their wild-type strain is due to a loss-of-function allele at the *PER1* locus (Roberg et al., 1997), which apparently is closely linked to (and possibly within the same transcriptional unit as) the $NADP^+$-dependent glutamate dehydrogenase-encoding *GDH1* gene (Courchesne and Magasanik, 1983). Remarkably, defects in *per1* are associated with a pleiotropic phenotype, including aberrant responses of various amino acid permeases (including Gap1) to environmental nitrogen signals (Courchesne and Magasanik, 1983). Although beyond the scope of this present study, it would be interesting to further characterize the potential synthetic interactions between EGOC/TORC1 and Per1/Gdh1 in future studies.

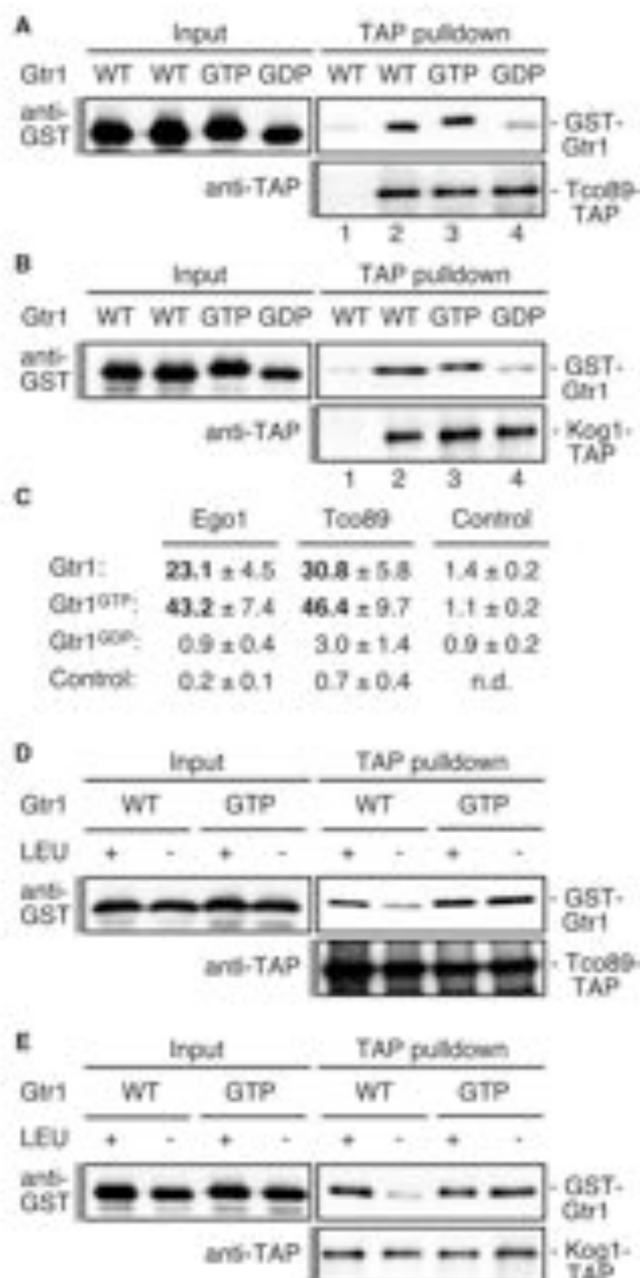


**Figure 4. Gtr1, Preferentially in Its GTP-Bound Form, Physically Interacts with TORC1**

(A and B) TORC1 subunits Tco89 and Kog1 preferentially interact with GTP-bound Gtr1$^{GTP}$. Wild-type control cells (A and B, lane 1) or wild-type cells expressing Tco89-TAP (A, lanes 2–4) or Kog1-TAP (B, lanes 2–4) were transformed with plasmids expressing GST-Gtr1 (A and B, lanes 1 and 2; WT), GST-Gtr1$^{GTP}$ (A and B, lane 3; GTP), or GST-Gtr1$^{GDP}$ (A and B, lane 4; GDP). Cell lysates (input) and TAP pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

(C) Gtr1 and Gtr1$^{GTP}$, but not Gtr1$^{GDP}$, specifically interact with Ego1 and Tco89 in two-hybrid assays. Possible interactions between Gtr1 variants (expressed from the pPR3-N prey vector) and Ego1 or Tco89 (both expressed from the pCabWT bait vector) were evaluated using the membrane-based split ubiquitin yeast two-hybrid system. Numbers represent mean β-galactosidase activities ± SD (in Miller units) from at least three different transformants for each pair of plasmids. Values that were at least 15-fold higher than the corresponding control (empty bait vector) are shown in bold.

(D and E) Gtr1-TORC1 interaction, but not Gtr1$^{GTP}$-TORC1 interaction, is sensitive to leucine starvation. Tco89-TAP (D) or Kog1-TAP (E) were precipitated from extracts prepared from wild-type cells coexpressing GST-Gtr1 or GST-Gtr1$^{GTP}$. Cells were either harvested in exponential growth phase 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-GST or anti-protein A (anti-TAP) antibodies as indicated.

Regarding the present study, loss of Gap1 did not reduce the cells' ability to recover from a rapamycin treatment (Figure 2C) and had no impact on TORC1 activity in cells grown on rich (YPD + 0.2% glutamine or SD-$NH_4^+$) or on poor (urea) nitrogen sources (Figure 2D). Taken together, these results, which were also reproducible in the completely unrelated KT1960 strain background (Pedruzzi et al., 2003; Stuart et al., 1994; data not shown), show that EGOC does not affect TORC1 via Gap1 sorting.

### Alternative Nucleotide-Bound States of Gtr1 and Gtr2 Are Required for Amino-Acid-Dependent Control of TORC1

To further examine the role of Gtr1 and Gtr2 in TORC1 regulation, we tested different combinations of nucleotide-restricted *GTR1* and *GTR2* alleles (Gao and Kaiser, 2006; Nakashima et al., 1999) for their effect on TORC1 activity and the cells' ability to recover from a rapamycin treatment. Expression of Gtr1$^{GTP}$ combined with Gtr2 or Gtr2$^{GDP}$ activated TORC1 (when compared to the corresponding heterodimers containing wild-type Gtr1) (Figure 3A). In contrast, expression of Gtr2$^{GTP}$ combined with Gtr1 inhibited TORC1 (when compared to the wild-type heterodimer). Moreover, expression of Gtr1$^{GDP}$, irrespective of the nucleotide-binding status of Gtr2, was dominant-negative, as it abolished TORC1-controlled Sch9 phosphorylation under all conditions (including cycloheximide treatment) (Figure 3A). Consistent with previous studies (Kim et al., 2008; Sancak et al., 2008), these observations demonstrate that GTP-loaded Gtr1 and GDP-loaded Gtr2 stimulate TORC1, whereas GDP-loaded Gtr1 and GTP-loaded Gtr2 inhibit TORC1.

Activation of TORC1 by expression of Gtr1$^{GTP}$ and by cycloheximide treatment was not additive, suggesting that amino-acid-dependent control of TORC1 may be controlled, at least in part, by the nucleotide-binding status of Gtr1 (Figure 3A). To explore this possibility further, we first sought to verify that cycloheximide treatment causes, as speculated, an increase in the pools of free intracellular amino acids. In prototrophic wild-type cells, we observed a significant accumulation of various amino acids within 5–15 min following cycloheximide treatment (Figure 3B). This effect (which was comparable in *gtr1Δ* cells; data not shown) and the corresponding activation of TORC1 were both observed to a similar extent in cells growing on YPD (+ 0.2% glutamine) and in cells growing on SD without amino acids (Figures 3B and S2), indicating that the accumulation of free amino acids following cycloheximide treatment does not depend on the uptake of extracellular amino acids. Interestingly, among the various amino acids, leucine appeared to accumulate most strongly and, hence, may play a particular role in TORC1

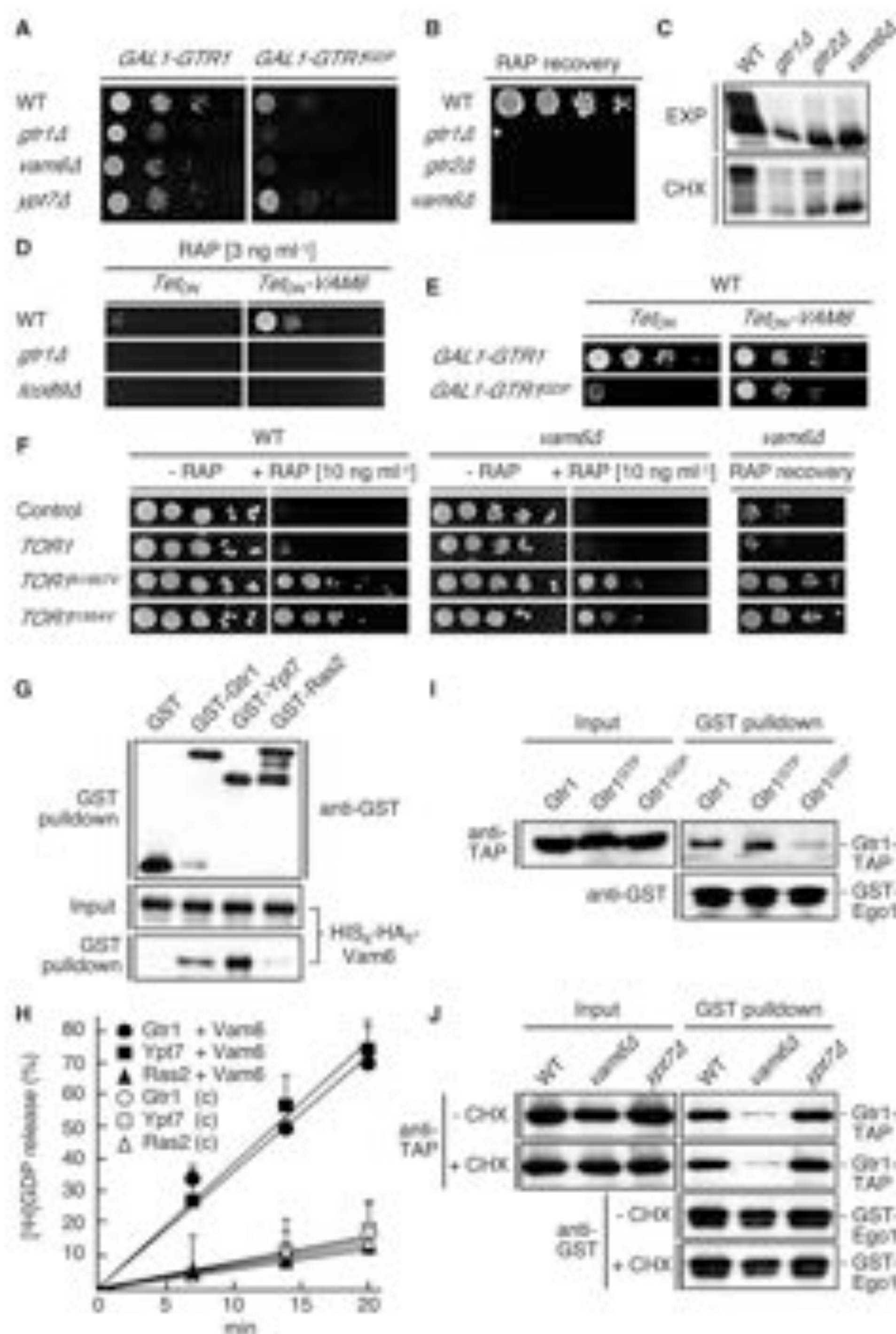


**Figure 5. Vam6 Is a Gtr1 Nucleotide Exchange Factor**

(A) Overproduction of Gtr1$^{GDP}$ results in a synthetic growth defect when combined with *gtr1Δ* or *vam6Δ*, but not when combined with *ypt7Δ*. Indicated strains expressing GTR1 or GTR1$^{GDP}$ from the galactose-inducible *GAL1* promoter were grown overnight to exponential phase and spotted on galactose-containing plates.

(B and C) Loss of Vam6, like loss of the EGOC subunits Gtr1 and Gtr2, causes a defect in recovery from a rapamycin-induced growth arrest (B) and decreases TORC1 activity (C). For experimental details, see Figure 1A.

(D) Overproduction of Vam6 from a doxycycline-inducible $Tet_{ON}$ promoter renders wild-type cells, but not *gtr1Δ* or *ivy?Δ* strains, resistant to low rapamycin concentrations. Strains carrying the indicated plasmids were grown overnight to exponential phase and spotted (in serial 10-fold dilutions) on plates containing 5 μg ml$^{-1}$ doxycycline and 3 ng ml$^{-1}$ rapamycin.

activation in yeast (Figure 3B). In line with this interpretation, we found TORC1 to be reversibly inactivated in response to leucine (and less pronouncedly in response to lysine or histidine) deprivation (Figure 3C). Notably, inhibition of TORC1 activity following leucine starvation could be partially suppressed by either treatment of cells with cycloheximide or overproduction of Gtr1$^{GTP}$ (Figures 3C and 3D); reactivation of TORC1 by readdition of leucine depended on Gtr1 but was abolished in the presence of Gtr1$^{GDP}$ (Figure 3D). Because overproduction of Gtr1$^{GTP}$ was unable to reverse TORC1 inactivation following $NH_4^+$ starvation (Figure S3), we infer from this data set that Gtr1, within an intact EGO complex, likely mediates at least part of the amino acid signaling to TORC1.

### Gtr1, Preferentially in Its GTP-Bound Form, Physically Interacts with TORC1

To examine whether Gtr1 may directly interact with TORC1, we coexpressed C-terminally TAP-tagged versions of the TORC1 subunits Tco89 or Kog1 (the yeast homolog of mammalian raptor; Loewith et al., 2002) and different GST-tagged Gtr1 variants. Using coimmunoprecipitation assays, we were able to demonstrate that both TORC1 subunits specifically and preferentially interacted with GTP-bound Gtr1, whereas the corresponding interactions with Gtr1$^{GDP}$ were close to background levels (Figures 4A and 4B). Employing a membrane based two-hybrid system, we confirmed that Gtr1 and, more efficiently, Gtr1$^{GTP}$, but not Gtr1$^{GDP}$, specifically associated with Tco89 as well as with the EGO complex subunit Ego1 (Figure 4C). Given the role of Gtr1 in mediating an amino acid signal to TORC1, we also evaluated whether amino acids regulate the interaction between Gtr1 and TORC1. We found that the Gtr1-TORC1 interaction was sensitive to leucine starvation, whereas Gtr1$^{GTP}$ remained associated with TORC1 under the same conditions (Figures 4D and 4E).

### Vam6 Is a Gtr1 Nucleotide Exchange Factor

Our findings above indicate that Gtr1 controls TORC1 function and that amino acid signals may impinge on this process by dictating the nucleotide-binding status of Gtr1. Accurate experimental determination of the Gtr1 nucleotide-binding status in vivo is not straightforward because Gtr1 and Gtr2 appear to be asymmetrically loaded with guanine nucleotides within heterodimeric complexes. To corroborate our model by alternative means, we therefore sought to isolate the Gtr1 GEF, which would allow us to more specifically modulate the nucleotide-binding status of Gtr1 within cells. Based on our finding that overproduction of Gtr1$^{GDP}$ was semidominant with respect to growth inhibition, we reasoned that loss of the Gtr1 GEF, like loss of Gtr1 (Figure 5A), should yield a strong synthetic growth defect when combined with a construct that allows accumulation of Gtr1$^{GDP}$. Consequently, we carried out a systematic, genome-wide synthetic dosage lethal (SDL) screen (Measday et al., 2005) in which we overproduced Gtr1$^{GDP}$ and tracked down corresponding synthetic growth defects. The two strongest positive hits in this screen included *gtr1Δ*—confirming the validity of our screening procedure—and *vam6Δ* (Figure 5A). The conserved Vam6 protein is a subunit of the homotypic fusion and vacuole protein sorting (HOPS/class C-Vps) complex in yeast (Caplan et al., 2001; Ostrowicz et al., 2008), which has recently also been suggested to control, via an unknown mechanism, TORC1 signaling in response to amino acids (Zurita-Martinez et al., 2007). Intriguingly, the HOPS complex is thought to facilitate the transition from tethering to *trans*-SNARE pairing during fusion at the vacuole, in part by Vam6-mediated nucleotide exchange on Ypt7, which is homologous to the mammalian Rab-7 GTPase (Wurmser et al., 2000). Combined with our data above (and the fact that Vam6, but not Ypt7, is required for cells to grow in the presence of elevated Gtr1$^{GDP}$ levels; Figure 5A), this led us to speculate that Vam6 may have a dual role in activation of both Ypt7 and Gtr1.

In accordance with a model in which Vam6 activates Gtr1, we found that loss of Vam6, like loss of Gtr1 (or other EGOC subunits), resulted in a defect in recovery from rapamycin-induced growth arrest, constitutively reduced TORC1 activity, and reduced cycloheximide-induced TORC1 hyperactivation (Figures 5B and 5C). Moreover, overproduction of Vam6

(E) Overproduction of Vam6 suppresses the semidominant growth defect conferred by overproduction of Gtr1$^{GDP}$. Wild-type cells expressing GTR1 or GTR1$^{GDP}$ under the control of the galactose-inducible GAL1 promoter were transformed with either an empty vector or a vector that expresses VAM6 from the doxycycline-inducible $Tet_{ON}$ promoter, grown overnight to exponential phase, and spotted (in serial 10-fold dilutions) on galactose- and doxycycline-containing plates.

(F) Expression of hyperactive TOR1$^{A1957V}$ or TOR1$^{I1954V}$ alleles renders wild-type and *vam6Δ* cells resistant to low rapamycin concentrations and enables *vam6Δ* cells to recover from a rapamycin-induced growth arrest. Wild-type and *vam6Δ* strains were transformed with an empty vector or vectors that express TOR1, TOR1$^{A1957V}$, or TOR1$^{I1954V}$ from their endogenous promoter and were spotted on plates containing, or not, 10 ng ml$^{-1}$ rapamycin and/or assayed for their ability to recover from a rapamycin-induced growth arrest as in Figure 1A.

(G) Gtr1 interacts with Vam6. Equal amounts of recombinant GST-Gtr1, GST-Ypt7, GST-Ras2, and GST alone, preloaded on glutathione-sepharose 4B resin, were incubated with extracts prepared from wild-type cells expressing $HIS_6$-$HA_3$-Vam6. Cell lysate (input) and GST pull-down fractions were then subjected to SDS-PAGE, and immunoblots were probed with anti-GST (top panel) or anti-HA (two lower panels) antibodies.

(H) Vam6 is a nucleotide exchange factor for Gtr1 and Ypt7, but not for Ras2. Recombinant GST-tagged GTP-binding proteins (i.e., 20 pmol of Gtr1 (●, ○), Ypt7 (■, □), or Ras2 (▲, △)) were preloaded with [$^3$H]GDP and incubated in the presence of $HIS_6$-$HA_3$-Vam6 (Vam6, filled symbols) or $HIS_6$-$HA_3$ (control [c], open symbols) that were purified from exponentially growing yeast. The time course of [$^3$H]GDP release is shown (in percent). Prior heat inactivation of Vam6 (10 min at 95°) reduced its GDP release activity toward Gtr1 to background levels (data not shown). Experiments were done in triplicate and expressed as mean ± SD.

(I) Gtr1 and Gtr1$^{GTP}$ interact strongly with Ego1. GST-Ego1 was precipitated from extracts prepared from wild-type cells coexpressing Gtr1-TAP, Gtr1$^{GTP}$-TAP, or Gtr1$^{GDP}$-TAP. Cell lysates (input) and GST pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

(J) Loss of Vam6, but not loss of Ypt7, abolishes the interaction between Gtr1 and Ego1. GST-Ego1 was precipitated from extracts prepared from wild-type, *vam6Δ*, or *ypt7Δ* cells coexpressing Gtr1-TAP. Cells were harvested in exponential growth phase prior to (– CHX) or after a 30 min cycloheximide (25 μg ml$^{-1}$) treatment (+ CHX). For details, see Figures 4A and 4B.

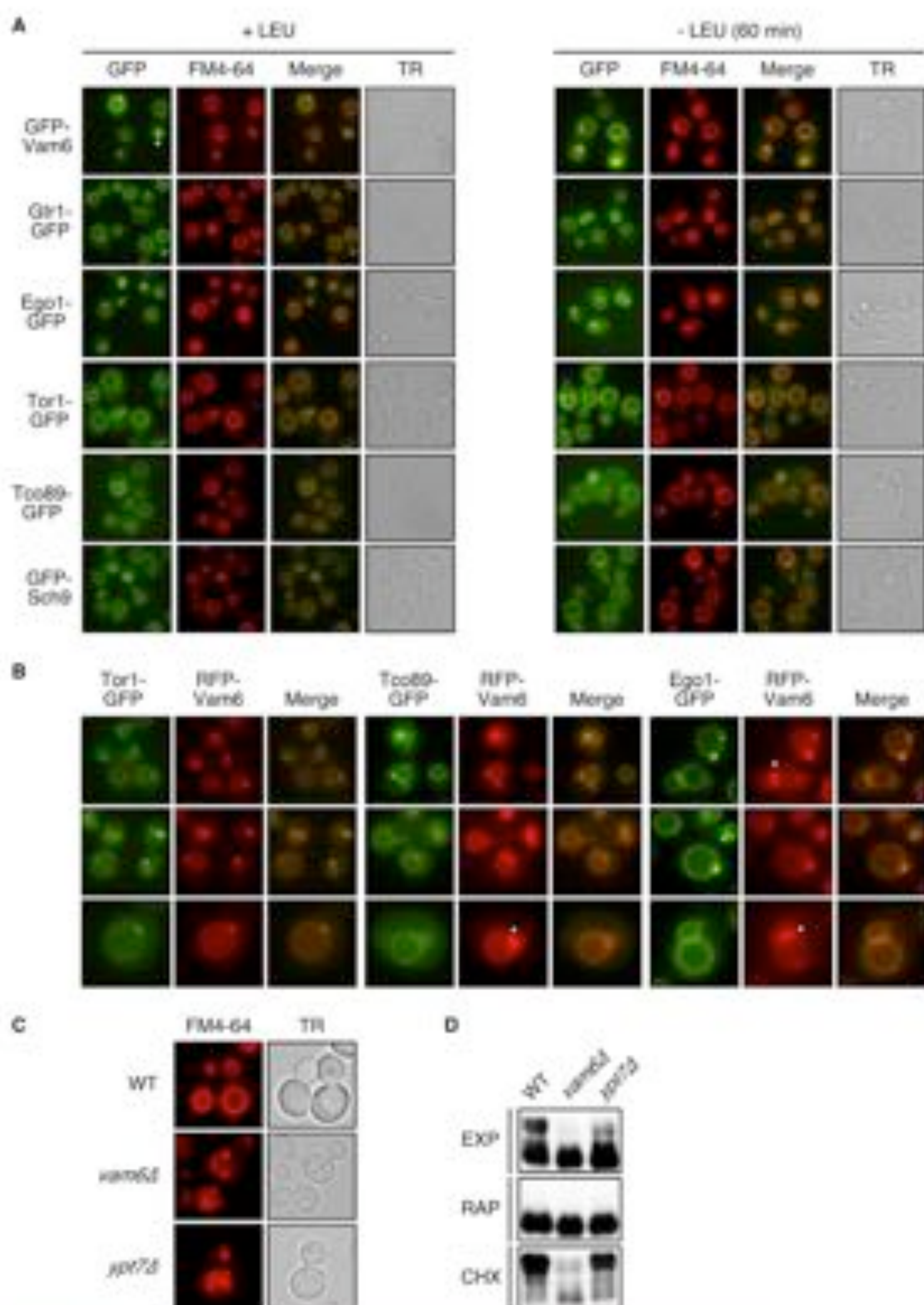


**Figure 6. TORC1 and EGO Complex Subunits Colocalize with Vam6 at Vacuolar and Endosomal Membranes**

(A) GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 all localize to the limiting membrane of the vacuole. Leucine (*leu2Δ*) auxotrophic wild-type cells expressing functional versions of GFP-fusion proteins either from a plasmid (GFP-Sch9) or from the endogenous chromosomal locus (Gtr1-GFP,

rendered wild-type cells, but not *gtr1Δ* or *tco89Δ* cells, resistant to low rapamycin concentrations (Figure 5D) and suppressed the semidominant growth defect resulting from Gtr1$^{GDP}$ overproduction (Figure 5E). Finally, expression of hyperactive *TOR1*$^{A1957V}$ and *TOR1*$^{I1954V}$ rendered rapamycin-sensitive *vam6Δ* cells as resistant to low rapamycin concentrations as wild-type cells and enabled *vam6Δ* cells to recover from a rapamycin-induced growth arrest (Figure 5F). Because these genetic experiments place Vam6 upstream of Gtr1, we determined whether Vam6 could interact with Gtr1. We found that Vam6 coprecipitated well with both GST-Gtr1 and the positive control GST-Ypt7; only very weakly with GST-Ras2, which is very closely related to Gtr1; and not at all with GST alone (Figure 5G). Further, Vam6 stimulated GDP release both from its known target Ypt7 and from Gtr1, but not from Ras2 (Figure 5H), indicating that Vam6 acts as a GEF for Gtr1 in vitro. Addition of nucleotide-free GST-Gtr2 did not significantly alter the Vam6-mediated GDP release on Gtr1 (data not shown). To explore whether Vam6 functions as a GEF for Gtr1 in vivo, we made use of the fact that Gtr1$^{GTP}$-TAP, but not Gtr1$^{GDP}$-TAP, specifically coprecipitates with GST-Ego1 (Figure 5I); hence, the level of Ego1-associated Gtr1 can be used to estimate the relative amount of Gtr1$^{GTP}$ within cells. Using this assay, we noted that loss of Vam6, but not loss of Ypt7, severely reduced the interaction between Gtr1 and Ego1 (both prior to and following cycloheximide treatment), indicating that Vam6 also functions as a GEF for Gtr1 in vivo (Figure 5J). Taken together, our results show that Vam6 regulates the nucleotide-binding status of Gtr1 both in vitro and in vivo and suggest that Vam6 may integrate amino acid signals to coordinate the control of TORC1 activity and vacuolar fusion events.

### TORC1 and EGO Complex Subunits Largely Colocalize with Vam6 at the Vacuolar Membrane

In line with previous reports (Araki et al., 2005; Gao and Kaiser, 2006; Jorgensen et al., 2004; Nakamura et al., 1997; Reinke et al., 2004; Sturgill et al., 2008; Urban et al., 2007), we found that GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 all localized predominantly at the vacuolar membrane in both exponentially growing and leucine-starved cells (Figure 6A). In addition, all GFP fusion proteins (except for GFP-Sch9) occasionally localized to punctate structures adjacent to the vacuole, which in the case of Vam6, Gtr1, and Ego1 may correspond to late endosomes (Cabrera et al., 2009; Gao and Kaiser, 2006). Notably, RFP-Vam6 colocalized with TORC1 and EGO complex subunits (i.e., with Tor1-GFP, Tco89-GFP, and Ego1-GFP) at the vacuolar and the presumed endosomal membranes (Figure 6B). Unlike Tor1-GFP, Tco89-GFP, or Ego1-GFP, however, RFP-Vam6 (and similarly GFP-Vam6) was found specifically enriched at the contact sites between two adjacent vacuoles (Figures 6A and 6B), which underscores the previously described role of Vam6 in vacuolar fusion. To exclude the possibility that the reduced TORC1 activity observed in *vam6Δ* cells is simply a result of their abnormal, fragmented vacuolar morphology (Figure 6C), we also measured TORC1 activity in *ypt7Δ* cells, which exhibit similarly fragmented vacuoles as *vam6Δ* cells (Figure 6C). The corresponding experiments clearly showed that only loss of Vam6, but not loss of Ypt7, seriously reduced TORC1 activity and prevented CHX-induced TORC1 activation, indicating that vacuolar fragmentation per se does not have an impact on TORC1 activity control (Figure 6D). Thus, together with the observation that the EGO complex is not required for vacuolar fusion (Dubouloz et al., 2005), it appears that Vam6 regulates two effector branches: one to control vacuolar fusion (via Ypt7) and one to control TORC1 (via the EGO complex subunit Gtr1).

### Conclusions

In conclusion, we have demonstrated that Vam6 activates Gtr1 and that Gtr1 and, by extension, the EGO complex act upstream of TORC1 to regulate its activity. Thus, our present work extends and confirms the evolutionarily conserved importance of Rag family GTPases as upstream regulators of TORC1. However, we were surprised that, in contrast to the situation in mammalian cells in which GTP-locked RagB apparently completely uncouples mTORC1 from signals derived from leucine availability (Sancak et al., 2008), GTP-locked Gtr1 had only a partial effect. Perhaps in yeast, there are other signaling routes by which amino acid availability is communicated to TORC1. Alternatively, it is possible that GTP loading must be followed by hydrolysis for Gtr1 to fully activate TORC1 in yeast; this later reaction would not be possible with the GTP-locked Gtr1 protein. In this context, it is also worth noting that mammalian cells express, due to alternative mRNA splicing, two RagB isoforms (RagB/RagB$^s$ and RagB$^l$), which are structurally significantly different from each other (Schürmann et al., 1995). It would, therefore, be of interest to also study the role of RagB$^l$ in mTORC1 regulation.

Finally, our finding that leucine deprivation did not appreciably affect the localization of GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 in yeast again contrasts with observations made in mammalian cells in which the Rag GTPases were proposed to mediate amino-acid-induced relocalization of mTOR within the endomembrane system to a compartment that contains the TORC1 activator Rheb (Sancak et al., 2008). Though this aspect of TORC1 regulation may have diverged during evolution—the budding yeast homolog of

Ego1-GFP, Tor1-GFP, and Tco89-GFP) or corresponding *leu2Δ vam6Δ* cells expressing functional GFP-Vam6 from a plasmid were labeled with the vacuolar membrane fluorescent dye FM4-64, and the localization of GFP fusion proteins was compared to FM4-64 staining by fluorescence microscopy. Cells were analyzed during exponential growth in leucine-containing SC medium (+ LEU) or following a 60 min leucine starvation period on SC medium lacking leucine (– LEU). TR, transmission.

(B) RFP-Vam6 colocalizes with Tor1-GFP, Tco89-GFP, and Ego1-GFP at vacuolar and presumed endosomal (green/red dots) membranes. In addition, RFP-Vam6 is specifically enriched at vacuolar fusion sites (arrows; see also GFP-Vam6 in A). Functional RFP-Vam6 was expressed from a plasmid. For details, see (A).

(C) Loss of Vam6 or Ypt7 results in fragmentation of vacuoles. Exponentially growing wild-type, *vam6Δ*, and *ypt7Δ* strains were stained with FM4-64 and analyzed as in (A). TR, transmission.

(D) Ypt7 is not required for TORC1 activation by cycloheximide (CHX). For experimental details, see Figure 1A.

Rheb is unlikely to perform a similar function in TORC1 activation (De Virgilio and Loewith, 2006a)—it will be most interesting to study whether Vam6 homologs are implicated in the control of TORC1 function by mediating nucleotide exchange on Rag GTPases in higher eukaryotes.

## EXPERIMENTAL PROCEDURES

### Strains, Growth Conditions, and Plasmids

The *S. cerevisiae* strains used in this study are listed in Table S1. Unless stated otherwise, prototrophic strains were progrown 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 YPD medium supplemented with 0.2% of glutamine and grown until they reached an $OD_{600}$ of 0.8. For amino acid deprivation experiments, strains that were specifically auxotrophic for one amino acid were grown to an $OD_{600}$ of 0.8 on complete synthetic medium (SC; i.e., SD plus all amino acids), washed twice, and resuspended in starvation medium (SC-Leu, SC-Lys, or SC-His lacking specifically one relevant amino acid). For nitrogen starvation, prototrophic cells were grown to an $OD_{600}$ of 1.0 on SD (without amino acids), washed twice, and resuspended in the same medium without ammonium sulfate. Low-quality nitrogen media were SD media, which contained 10 mM urea or proline instead of ammonium sulfate. The plasmids used in this study are listed in Table S2.

### Sch9 Phosphorylation Analyses

To analyze $Sch9^{T737A}$-$HA_5$ C-terminal phosphorylation, we used the chemical fragmentation analysis as described previously (Urban et al., 2007; Wanke et al., 2008). For quantifications of Sch9 phosphorylation, NTCB-cleaved extracts were separated by 7.5% SDS-PAGE followed by immunoblotting with anti-HA antibody 12CA5. The anti-HA antibody was detected with far-red fluorescent Alexa Fluor 680 dye-labeled secondary anti-mouse antibody (Invitrogen, A21057), and fluorescence intensity was measured using the Odyssey Infrared Imaging System (LI-COR).

### GDP Release Assay

To assay GDP release, 20 pmol of bacterially expressed GST-tagged G proteins (i.e., GST-Gtr1, GST-Ypt7, and GST-Ras2) were preloaded by incubating with 40 pmol 5',8'-[$^3$H]GDP (32.8 Ci mmol$^{-1}$; NEN) in preload buffer (20 mM HEPES [pH 7.2], 20 mM KOAc, 1 mM DTT, 5 mM EDTA, 1 μg μl$^{-1}$ BSA) for 15 min at 30°C as described (Jones et al., 2000). At the end of the incubation, samples were placed on ice, and $MgCl_2$ was added to 10 mM. Reactions were carried out in 50 μl containing 20 mM HEPES (pH 7.2), 5 mM $Mg(OAc)_2$, 0.5 mM GTP, 0.5 mM GDP, 1 mM DTT, 0.4 mg ml$^{-1}$ BSA, and $HIS_6$-$HA_3$-Vam6 or $HIS_6$-$HA_3$, both purified from exponentially growing yeast. Exchange reactions were initiated by the addition of 10 pmol of the preloaded G proteins. Incubations were carried out at 30°C for varying periods of time, as noted. At intervals, 5 μl samples were removed, added to 3 ml of ice-cold wash buffer (20 mM Tris-HCl [pH 7.5], 20 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT), and filtered through nitrocellulose filters, which were then washed twice with 3 ml of ice cold wash buffer. Radioactivity bound to filters was quantified by liquid scintillation spectrometry using Filtron-X (National Diagnostics, LS-201) scintillation fluid. In all experiments, initial values were ~2–4 × $10^3$ dpm μl$^{-1}$.

### Miscellaneous

Coimmunoprecipitation experiments were essentially done as described (Dubouloz et al., 2005; Loewith et al., 2002). Tco89-TAP and Kog1-TAP were purified from lysates with magnetic beads (Invitrogen) that were precoated overnight in PBS with 1 mg ml$^{-1}$ IgG (Sigma) in the presence of 1 M $(NH_4)_2SO_4$ to facilitate the binding. For quantifications of free amino acids, cells (about 10 $OD_{600}$) were harvested by filtration, washed three times, and resuspended in distilled water. Following boiling (15 min) and centrifugation (10 min at 13,000 rpm), free amino acids were quantified in the supernatant by pulsed electrochemical detection after separation by anion exchange chromatography with an AAA-direct Dionex Amino Acid Analyzer, using a sodium acetate gradient to increase the ionic strength.

## SUPPLEMENTAL DATA

Supplemental Data include two tables and three figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00474-2.

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## REFERENCES

Araki, T., Uesono, Y., Oguchi, T., and Toh, E.A. (2005). LAS24/KOG1, a component of the TOR complex 1 (TORC1), is needed for resistance to local anesthetic tetracaine and normal distribution of actin cytoskeleton in yeast. Genes Genet. Syst. 80, 325–343.

Beck, T., and Hall, M.N. (1999). The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402, 689–692.

Beugnet, A., Tee, A.R., Taylor, P.M., and Proud, C.G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. Biochem. J. 372, 555–566.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132.

Cabrera, M., Ostrowicz, C.W., Mari, M., LaGrassa, T.J., Reggiori, F., and Ungermann, C. (2009). Vps41 phosphorylation and the Rab Ypt7 control the targeting of the HOPS complex to endosome-vacuole fusion sites. Mol. Biol. Cell 20, 1937–1948.

Caplan, S., Hartnell, L.M., Aguilar, R.C., Naslavsky, N., and Bonifacino, J.S. (2001). Human Vam6p promotes lysosome clustering and fusion in vivo. J. Cell Biol. 154, 109–122.

Courchesne, W.E., and Magasanik, B. (1983). Ammonia regulation of amino acid permeases in Saccharomyces cerevisiae. Mol. Cell. Biol. 3, 672–683.

De Craene, J.O., Soetens, O., and André, B. (2001). The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J. Biol. Chem. 276, 43939–43948.

De Virgilio, C., and Loewith, R. (2006a). Cell growth control: little eukaryotes make big contributions. Oncogene 25, 6392–6415.

De Virgilio, C., and Loewith, R. (2006b). The TOR signalling network from yeast to man. Int. J. Biochem. Cell Biol. 38, 1476–1481.

Dubouloz, F., Deloche, O., Wanke, V., Cameroni, E., and De Virgilio, C. (2005). The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol. Cell 19, 15–26.

Gao, M., and Kaiser, C.A. (2006). A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. Nat. Cell Biol. 8, 657–667.

Gao, X.D., Wang, J., Keppler-Ross, S., and Dean, N. (2005). *ERS1* encodes a functional homologue of the human lysosomal cystine transporter. FEBS J. 272, 2497–2511.

Grenson, M. (1983). Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast Saccharomyces cerevisiae. Eur. J. Biochem. 133, 135–139.

Hirose, E., Nakashima, N., Sekiguchi, T., and Nishimoto, T. (1998). RagA is a functional homologue of S. cerevisiae Gtr1p involved in the Ran/Gsp1-GTPase pathway. J. Cell Sci. 111, 11–21.

Jauniaux, J.C., and Grenson, M. (1990). GAP1, the general amino acid permease gene of Saccharomyces cerevisiae. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. 190, 39–44.

Jones, S., Newman, C., Liu, F., and Segev, N. (2000). The TRAPP complex is a nucleotide exchanger for Ypt1 and Ypt31/32. Mol. Biol. Cell 11, 4403–4411.

Jorgensen, P., Rupes, I., Sharom, J.R., Schneper, L., Broach, J.R., and Tyers, M. (2004). A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. Genes Dev. 18, 2491–2505.

Kaiser, C.A., Chen, E.J., and Losko, S. (2002). Subcellular fractionation of secretory organelles. Methods Enzymol. 351, 325–338.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nat. Cell Biol. 10, 935–945.

Kuranda, K., Leberre, V., Sokol, S., Palamarczyk, G., and François, J. (2006). Investigating the caffeine effects in the yeast Saccharomyces cerevisiae brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. Mol. Microbiol. 61, 1147–1166.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell 10, 457–468.

Measday, V., Baetz, K., Guzzo, J., Yuen, K., Kwok, T., Sheikh, B., Ding, H., Ueta, R., Hoac, T., Cheng, B., et al. (2005). Systematic yeast synthetic lethal and synthetic dosage lethal screens identify genes required for chromosome segregation. Proc. Natl. Acad. Sci. USA 102, 13956–13961.

Nakamura, N., Hirata, A., Ohsumi, Y., and Wada, Y. (1997). Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 272, 11344–11349.

Nakashima, N., Noguchi, E., and Nishimoto, T. (1999). Saccharomyces cerevisiae putative G protein, Gtr1p, which forms complexes with itself and a novel protein designated as Gtr2p, negatively regulates the Ran/Gsp1p G protein cycle through Gtr2p. Genetics 152, 853–867.

Nikko, E., Marini, A.M., and André, B. (2003). Permease recycling and ubiquitination status reveal a particular role for Bro1 in the multivesicular body pathway. J. Biol. Chem. 278, 50732–50743.

Ostrowicz, C.W., Meiringer, C.T., and Ungermann, C. (2008). Yeast vacuole fusion: a model system for eukaryotic endomembrane dynamics. Autophagy 4, 5–19.

Pedruzzi, I., Dubouloz, F., Cameroni, E., Wanke, V., Roosen, J., Winderickx, J., and De Virgilio, C. (2003). TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into $G_0$. Mol. Cell 12, 1607–1613.

Reinke, A., Anderson, S., McCaffery, J.M., Yates, J., III, Aronova, S., Chu, S., Fairclough, S., Iverson, C., Wedaman, K.P., and Powers, T. (2004). TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. J. Biol. Chem. 279, 14752–14762.

Reinke, A., Chen, J.C., Aronova, S., and Powers, T. (2006). Caffeine targets TOR complex I and provides evidence for a regulatory link between the FRB and kinase domains of Tor1p. J. Biol. Chem. 281, 31616–31626.

Roberg, K.J., Bickel, S., Rowley, N., and Kaiser, C.A. (1997). Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. Genetics 147, 1569–1584.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496–1501.

Sarbassov, D.D., Ali, S.M., and Sabatini, D.M. (2005). Growing roles for the mTOR pathway. Curr. Opin. Cell Biol. 17, 596–603.

Schürmann, A., Brauers, A., Massmann, S., Becker, W., and Joost, H.G. (1995). Cloning of a novel family of mammalian GTP-binding proteins (RagA, RagBs, RagB1) with remote similarity to the Ras-related GTPases. J. Biol. Chem. 270, 28982–28988.

Sekiguchi, T., Hirose, E., Nakashima, N., Ii, M., and Nishimoto, T. (2001). Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. J. Biol. Chem. 276, 7246–7257.

Springael, J.Y., and André, B. (1998). Nitrogen-regulated ubiquitination of the Gap1 permease of Saccharomyces cerevisiae. Mol. Biol. Cell 9, 1253–1263.

Stuart, J.S., Frederick, D.L., Varner, C.M., and Tatchell, K. (1994). The mutant type 1 protein phosphatase encoded by glc7-1 from Saccharomyces cerevisiae fails to interact productively with the GAC1-encoded regulatory subunit. Mol. Cell. Biol. 14, 896–905.

Sturgill, T.W., Cohen, A., Diefenbacher, M., Trautwein, M., Martin, D.E., and Hall, M.N. (2008). TOR1 and TOR2 have distinct locations in live cells. Eukaryot. Cell 7, 1819–1830.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., et al. (2007). Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol. Cell 26, 663–674.

Wanke, V., Cameroni, E., Uotila, A., Piccolis, M., Urban, J., Loewith, R., and De Virgilio, C. (2008). Caffeine extends yeast lifespan by targeting TORC1. Mol. Microbiol. 69, 277–285.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell 124, 471–484.

Wurmser, A.E., Sato, T.K., and Emr, S.D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. J. Cell Biol. 151, 551–562.

Zurita-Martinez, S.A., Puria, R., Pan, X., Boeke, J.D., and Cardenas, M.E. (2007). Efficient Tor signaling requires a functional class C Vps protein complex in Saccharomyces cerevisiae. Genetics 176, 2139–2150.

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Regulation of TORC1 pathway by amino acids in *S. cerevisiae*
bibliographic search, protocol set-up and writing, oral presentations
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scientific watch/bibliographic search
project monitoring : experimental protocol set-up and writing, scientific report writing, innovative proposals to identified problems, oral presentations
UPLC/MS for quantification of small active molecules in biological samples, enzymatic reaction follow-up; chemical library screening for pharmaceutical companies

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report writing
epifluorescence microscopy, multicopy suppressor screen, ChIP, qPCR

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Report writing
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Binda, M., Péli-Gulli, M.P., Bonfils, G., Panchaud, N., De Virgilio C. (2010). An EGOcentric view of TORC1 signaling. Cell Cycle 9(2):221-2

Binda, M., Péli-Gulli, M.P., Bonfils, G., Panchaud, N., Urban, J., Sturgill, T.W., Loewith, R., and De Virgilio, C. (2009). The Vam6 GEF controls TORC1 by activating the EGO complex. Mol Cell *35*, 563-573.

**Attendance to meetings:**

April 2011: Levures Modèle et Outils X, Toulouse, France; oral and poster presentation
September 2011: TOR, PI3K, and Akt- 20 years on, Basel, Switzerland
July 2011: 25th international conference on yeast genetics and molecular biology, Olsztyn-Kortowo, Poland; poster presentation
September 2010: Swiss Yeast Meeting
September 2010: Levures Modèle et Outils IX, Strasbourg, France; poster presentation

**Others:**

Computer skills: Microsoft office, Photoshop, Canvas etc.
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