Université de Fribourg (Suisse)
Département de Biologie
Unité de Biochimie

Regulation and Architecture of the *Saccharomyces cerevisiae* EGO Complex, which controls growth via TORC1

THESE

Présentée à la Faculté des Sciences de l'Université de Fribourg (Suisse)
pour l'obtention du titre doctor rerum naturalium

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numéro de thèse: 1867
Editions Fricopy
2014

Acceptée par la Faculté des Sciences de l'Université de Fribourg (Suisse) sur la proposition de:

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Fribourg, le 3 octobre 2014

Le Directeur de Thèse: Le Doyen:

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“L'homme fait à tous les instants des déclarations définitives sur la vie, l'homme et l'art,
et ne sait pas plus que le champignon ce qu'est la vie, l'homme et l'art.”
Jean Arp

List of abbreviations:

AAP: amino acid permeases
ADP: adenosine diphosphate
AMP: adenosine monophosphate
ATP: adenosine triphosphate
ATPase: adenosine triphosphatase
BCAA: branched chain amino acids
cAMP: cyclic adenosine monophosphate
EGOC: exit from rapamycin-induced growth arrest
GAAC: general amino acid contro pathway
GAP: GTPase activating protein
GATOR: GAP activity toward Rags
GDI: guanine dissociation inhibitors
GDP: guanosine diphosphate
GEF: guanine nucleotide exchange factors
GPCR: G protein coupled receptors
GTP: guanosine triphosphate
GTPase: guanosine triphosphatase
LeuRS: leucyl t-RNA synthetase
mRNA: messenger RNA
mTORC1: mammalian target of rapamycin complex 1
NCR: nitrogen catabolite repression pathway
ORF: open reading frame
P_i: inorganic phosphate
PKA: protein kinase A
PP2A: protein phosphatase 2 A
Rag: Ras-related GTP binding protein
Ribi: ribosome biogenesis
RNA: ribonucleic acid
RP: ribosomal protein
RTG: retrograde transport pathway
SCF: Skp, Cullin, F-box
SEAC: Seh1-associated complex
SEACAT: SEAC subcomplex activating TORC1 signaling
SEACIT: SEAC subcomplex inhibiting TORC1 signaling
TAP: tandem affinity purificaiton
TCA: tricarboxylic acid cycle
TOR: target of rapamycin
TORC1: target of rapamycin complex 1
v-ATPase: vacuolar ATPase

Summary

Living organisms need to respond to environmental changes in order to survive. Unicellular as well as multicellular organisms have developed various mechanisms to sense their environment and to react properly to changes. In eukaryotes, the structurally and functionally conserved Target Of Rapamycin Complex 1 (TORC1) is a central regulator of cell growth that responds to hormonal, growth factor, energy and nutritional stimuli. TORC1 promotes protein synthesis, transcription, translation initiation, mRNA stability, ribosome biogenesis, etc., and inhibits stress response programs and catabolic processes such as autophagy. Amino acids, especially branch-chained amino acids like leucine, are primordial TORC1 nutritional cues that cannot be compensated for by any other nutrient. The highly conserved family of Rag GTPases is required to communicate amino acid levels to TORC1. In higher eukaryotes, RagA or RagB forms a heterodimer with RagC or RagD, whereas yeast Gtr1 heterodimerizes with Gtr2. The RAG GTPase heterodimer is able to stimulate TORC1 activity when RagA/B/Gtr1 is bound to GTP and RagC/D/Gtr2 to GDP. Rag GTPases require a protein complex coined Ragulator in higher eukaryotes and EGOC in yeast to localize to the lysosome/vacuole and to properly signal amino acid sufficiency to TORC1.

The aim of this thesis was to understand how amino acids regulate the EGOC, in particular how they modulate the nucleotide loading status of the Gtr1/Gtr2 heterodimer. In the first chapter we identified Iml1 as a negative regulator for Gtr1. Iml1 act as a GAP and thus stimulates GTP hydrolysis activity of Gtr1. *In vivo* Iml1 interaction with Gtr1 depends on two other partners, Npr2 and Npr3, and is transiently stimulated upon amino acid deprivation. Hence, Iml1 in association with Npr2 and Npr3 prevents Gtr1 from activating TORC1 in response to amino acid deprivation. As Iml1, Npr2 and Npr3 were previously found to be part of the larger SEA complex, we decided to rename the Iml1-Npr2-Npr3 subcomplex SEACIT (for SEAC subcomplex Inhibiting TORC1 signaling). We found that the other members of the SEA complex, Seh1, Sec13, Sea2, Sea3 and Sea4, form a second subcomplex that acts upstream and negatively toward SEACIT. We therefore renamed it SEACAT (for SEAC subcomplex Activating TORC1 signaling).

In the second chapter we investigated the role of two uncharacterized yeast proteins as potential orthologs of HBXIP and C7orf59, two recently identified components of Ragulator. In higher eukaryotes, HBXIP and C7orf59 are required to confer to

Ragulator GEF activity toward RagA/B. This led us to identify Ycr075w as a novel component of the EGO complex. Our *in vitro* assays did not allow us to show that the expanded EGOC (harbouring Ego1, Ycr075W [now coined Ego2], and Ego3) had any GEF activity toward Gtr1. Nevertheless Ego2 is required for the localization of Gtr1 and Gtr2 at the vacuolar membrane and for the proper activation of TORC1.

Finally, in chapter three, we examined the effect on TORC1 of a small molecule identified in a high throughput screen for rapalogs and delineated polyamines as potential TORC1 inhibitors with anti-ageing effects.

Résumé

Au cours de sa vie, tout organisme est inévitablement confronté à des changements de son environnement. Dès lors, sa survie dépend de son aptitude à sentir de telles variations environnementales et à moduler son état de croissance en conséquence. Chez les eucaryotes uni- et multicellulaires, le complexe protéine kinase TORC1 (Target Of Rapamycin) joue un rôle capital dans la régulation de la croissance cellulaire. En réponse à des stimuli de diverses natures (hormones, facteurs de croissance, nutriments), TORC1 est activé et engage les processus de transcription, de stabilité des ARN messagers, de biogenèse des ribosomes, d'initiation de la traduction et de synthèse protéique. Parallèlement, il inhibe les programmes de réponse au stress ainsi que les processus cataboliques tels que l'autophagie.

Parmi les nutriments, les acides aminés et plus spécifiquement les acides aminés ramifiés comme la leucine, représentent un signal essentiel à l'activation de TORC1. Une famille de petites GTPases nommée Rag contribue à informer TORC1 de la disponibilité des acides aminés. Chez les eucaryotes supérieurs, RagA ou RagB forme un hétérodimère avec RagC ou RagD, alors que, chez la levure, Gtr1 forme un hétérodimère avec Gtr2. Lorsque RagA ou B / Gtr1 est lié au GTP et RagC ou D / Gtr2 est lié au GDP, l'hétérodimère conduit à l'activation de TORC1. La localisation des Rag et de TORC1 au lysosome/vacuole requiert un complexe de protéines nommé Ragulator chez les eucaryotes supérieurs et EGO complexe chez la levure.

Durant ce travail de thèse, nous avons cherché à comprendre comment les acides aminés régulent le complexe EGO et, en particulier, comment ils modulent l'association des Rag GTPases Gtr1 et Gtr2 avec leurs nucléotides. Dans le premier chapitre, nous avons identifié Iml1 comme régulateur négatif de Gtr1. En tant que GAP, Iml1 stimule la capacité de Gtr1 à hydrolyser le GTP. *In vivo,* son interaction avec Gtr1 dépend de deux autres partenaires, Npr2 et Npr3 et est stimulée de manière transitoire par une privation d'acides aminés. Ainsi donc, Iml1, associé à Npr2 et Npr3, empêche Gtr1 d'activer TORC1 lorsque les acides aminés viennent à manquer. Iml1, Npr2 et Npr3 ont été précédemment identifiés comme faisant partie d'un complexe protéique plus grand nommé SEA complexe. Nous avons donc renommé ce sous-complexe SEACIT (pour SEA sous-Complexe Inhibant TORC1). Nous avons ensuite découvert que les autres membres du SEA complexe, Seh1, Sec13, Sea2, Sea3 and Sea4, quant à eux, agissent en amont de SEACIT et de

manière opposée. Pour cette raison, nous avons appelé ce sous-groupe SEACAT (pour SEA sous-Complexe Activant TORC1).

Dans le deuxième chapitre, nous avons étudié la fonction de deux protéines de levure, possibles orthologues de HBXIP et C7orf59, deux composants du Ragulator récemment identifiés. Chez les eucaryotes supérieurs, ces derniers confèrent au Ragulator une activité GEF envers RagA/B. Nous avons montré que Ycr075w est un nouveau composant du complexe EGO. Nos essais *in vitro* n'ont pas permis de déceler chez cette version étendue du complexe EGO une activité GEF envers Gtr1. Néanmoins, en assurant la localisation correcte de Gtr1 et Gtr2 à la membrane vacuolaire, Ycr075w (nommé Ego2) s'avère nécessaire à l'activation de TORC1.

Finalement, dans le troisième chapitre, nous avons examiné l'effet sur TORC1 d'une molécule découverte dans un crible à large échelle entrepris pour rechercher de nouveaux inhibiteurs de TORC1 (rapalogues). Nous avons également défini les polyamines comme inhibiteur potentiel de TORC1 avec une possible application dans le traitement des maladies prolifératives ou liées à l'âge.

Introduction

Signaling Pathways

The survival of any living organism depends on its ability to respond to environmental changes in an appropriate manner in order to maintain homeostasis. At a cellular level this involves a variety of mechanisms allowing sensing of nutrients, stress and hormones. The cell has to integrate these different signals to then make appropriate decisions regarding its fate (*i.e.* to grow, proliferate, differentiate, enter quiescence, or die)(Bahn et al., 2007). Sensing mechanisms typically involve the specific reception of a signal (e.g., at the cell surface), its transduction inside the cell, and a response that can occur at the transcriptional, translational, post-translational, or metabolic level (Zaman et al., 2008). Between the primary sensor and the final effectors, signal transduction involves a multitude of actors that can modulate the signal. Crosstalk between different pathways is also essential. Hence cells possess hubs that are able to integrate multiple signals and subsequently activate or inhibit different targets. Signal transduction and control of cellular responses are generally tightly regulated processes and their deregulation can lead to dramatic consequences such as cancer, diabetes and autoimmune diseases. Signal transduction can be achieved by different mechanisms that involve changes in the conformation, stability, or localization of proteins. I will briefly give an overview of some of these key mechanisms in the following paragraphs.

i) Phosphorylation

One of the best-understood mechanisms in signal transduction is protein phosphorylation, in which a phosphate group is added covalently to a serine, threonine, or tyrosine. It has been estimated that one third of all proteins are phosphorylated at least once during their life cycle (Zolnierowicz and Bollen, 2000). Protein kinases are the enzymes responsible for protein phosphorylation. They use adenosine triphosphate (ATP) as donor molecules and ensure their specificity in part by recognizing consensus sequences surrounding the target residue (BURNETT and KENNEDY, 1954). A consequence of a phosphorylation event is typically a conformational change in the target protein that can affect its activity, turnover, localization, or ability to interact with other proteins. Protein phosphatases can remove phosphate groups from a protein, thus antagonizing the action of protein kinases (Efeyan et al., 2014; STARK, 1996). As many phosphorylation sites can be

present on a single protein and as different kinases can potentially target a given site, protein phosphorylation provides a means to fine-tune regulation of target proteins (Efeyan et al., 2012a; Roach, 1991).

ii) Ubiquitination

Ubiquitination is another very important post-translational modification for signaling pathways. It involves the addition of a small polypeptide (76 amino-acids), coined ubiquitin, to a lysine residue of a target protein (Finley et al., 2012). Ubiquitin itself contains seven lysines that can be further ubiquitinated in a process called polyubiquitinaiton. In some cases ubiquitination is perceived as signal for re-localization of the target protein (*i.e.* endocytosis), but in many cases it acts as a tag for specific degradation by the 26S proteasome (Pickart, 2001). Notably, many ubiquitination events require first a phosphorylation that will change the conformation of the target protein and thereby expose a binding surface for the ubiquitination machinery (Finley et al., 2012). A combination of three enzymes is required for ubiquitination: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3), which mediates substrate specificity (Bohn et al., 2007; Pickart, 2001).

iii) GTPases

Guanosine Tri-Phosphatases (GTPases) are also crucial elements within cell signaling cascades. These enzymes contain a G domain that is able to bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and inorganic phosphate (P_i) in a reaction that requires magnesium (Mg^{2+}) (Bourne et al., 1990; Sancak et al., 2010). Hydrolysis of GTP to GDP triggers a conformational change that switches the GTPase from an active form (usually GTP-bound) to an inactive form (usually GDP-bound) (Bourne et al., 1990). The G domain has an α-helix/β-sheet structure with a conserved motif that binds the guanine base and a P-loop motif responsible for the binding of Mg^{2+} and the β-phosphate in GTP (Scheffzek and Ahmadian, 2005). Two additional regions from the G domain, switch I and switch II, interact with the γ phosphate in GTP; when this phosphate is removed by hydrolysis, switch I and II undergo drastic changes in their structure (Scheffzek and Ahmadian, 2005). Due to this conformational change, GTPases act as molecular

switches in signaling pathways. A change from the GDP-bound “OFF” state to the GTP-bound “ON” state allow GTPases to interact with specific effector proteins and thereby transmit a signal. GTPases can be divided into three families: the heterotrimeric G proteins, the superfamily of small GTPases, and the G proteins activated by nucleotide dependent dimerization (GADs)

a) Heterotrimeric G proteins

Heterotrimeric G proteins are composed of an α-subunit, containing the G domain, and two smaller domains coined β and γ subunit (Huang and Manning, 2008; Siderovski and Willard, 2005). The complex is attached to membranes via lipid anchors present in the α and γ subunit. Typically, these GTPases are associated in their inactive form (*i.e.* GDP bound) with seven transmembrane domain receptors named G Protein Coupled Receptors (GPCR)(Bar-Peled et al., 2013; Gilman, 1987). Binding of the signaling ligand by the GPCR provokes a conformational change that promotes GEF activity of the GPCR on the GTPase. Once loaded with GTP, the α-subunit dissociates from the β- and γ-subunits. Both the α-subunit and the heterodimeric βγ subunit can then activate or inhibit effector proteins. Following GTP hydrolysis, the heterotrimeric complex can be formed again and then re-associate with the GPCR (Siderovski and Willard, 2005). A very well described example of a heterotrimeric G protein effector is the adenylyl cyclase, which converts ATP to cAMP and subsequently activates, among others, PKA (D'Souza and Heitman, 2001). In metazoans, heterotrimeric G proteins and their cognate GPCR are used for sensing and transmitting light, odors, and flavors. They also have a central role in signal transduction in the nervous system. Numerous natural or synthetic drugs have been described that interfere with the function of GPCRs.

b) Small GTPases

The Ras GTPase superfamily, named after its founding member Ras, comprises more than 150 members. They are small globular proteins (25-30 kDa) with a G-domain that is structurally related to the G_{α} subunit of heterotrimeric G proteins. The Ras superfamily can be subdivided into 5 sub-groups: (i) the Ras family of proteins that play important roles in cellular signaling, (ii) the Rho family of proteins that are mainly involved in actin reorganization, (iii) the Rab family proteins that regulate

vesicular trafficking, (iv) the Ran family proteins responsible for nucleo-cytoplasmic transport of RNA and proteins, and (v) Arf family proteins facilitating vesicle coat formation in the exocytic and endocytic pathway (Wennerberg et al., 2005). The majority of small GTPases are associated with membranes, either via interactions with membrane-anchored partners or directly via a lipid modification. GTPases harboring a C-terminal CAAX motif (C = Cys, A = aliphatic, X = any amino acid), like the majority of Rho and Ras family proteins, can be modified by two types of cysteine prenylation, namely farnesylation or geranyl-geranylation. Rab family proteins harbor a different type of motif (*e.g.*, CC, CXC, CCX, CCXX, or CCXXX) that can also be geranyl-geranylated (Wennerberg et al., 2005). Some members of Arf family proteins are targeted to membranes via N-terminal myristoylation (Wennerberg et al., 2005).

c) G proteins activated by nucleotide dependent dimerization

G proteins activated by nucleotide dependent dimerization (GAD) are a poorly characterized family of G proteins that form dimers in a nucleotide dependent manner, with GTP promoting the interaction between their G domains. When dimerized, these GTPases reciprocally provide residues to each other to enhance their enzymatic activity (Gasper et al., 2009). The best-known examples of this family are the components of the Signal Recognition Particle (SRP) and its receptor (SR), which co-translationally target proteins to membrane compartments. The structure of core components of these complexes has been elucidated for instance in bacteria for the GTPases Ffh and FtsY, which dimerize in a head-to-tail manner (Focia et al., 2004). In this example two different GTPases form the dimer. However, two identical GTPases can also homo-dimerize, as is the case for human LRRK2, dynamin or bacterial MnmE (Gasper et al., 2009; Picard et al., 2014).

As most of the GTPases have a very slow intrinsic activity and high affinity for GTP/GDP, regulatory proteins are required to speed up the reaction to a physiologically relevant rate. GTPase Activating Proteins (GAP) accelerate hydrolysis of the GTP, Guanine nucleotide Exchange Factors (GEF) promote the exchange of nucleotides and Guanine Dissociation Inhibitors (GDI) can re-localize lipid-modified GTPases (Fig.1).

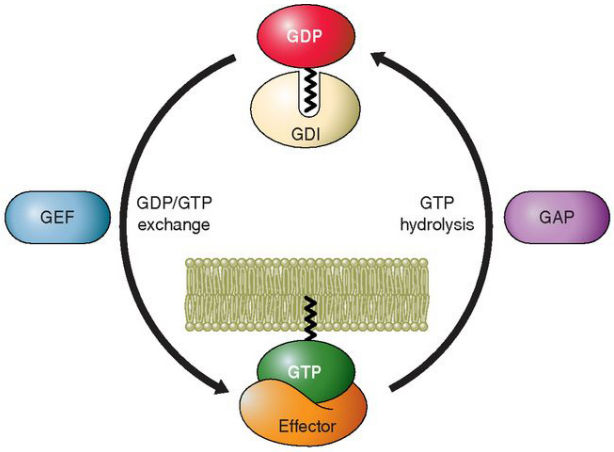

Fig. 1 The GTPase cycle. GTPases switch between a GTP-bound state and GDP-bound state. GEFs stimulate nucleotide exchange and thereby activate GTPases. GAPs assist GTP hydrolysis and thereby promote inactivation of GTPases. Localization of some GTPases is regulated by GDIs. Taken from (Cherfils and Zeghouf, 2013; Picard et al., 2014).

d) GTPase activating proteins

GAPs accelerate hydrolysis of GTP by providing critical residues to stabilize the transition state between GTP and GDP and/or by stabilizing the GTPase switch regions. Typically GAPs are composed of multiple domains, one of them (usually with a size of 100-300 amino acids) being responsible for the GAP activity (Picard et al., 2014; Scheffzek et al., 1998). GAPs preferentially interact with GTP bound GTPases. However the nature of these interactions are transient. In the presence of aluminium fluoride (AlF_x , x = 3 or 4), GAP-GTPase interactions can be stabilized. In complex with GDP, AlF_x mimics the transition state from GTP to GDP, which was a key discovery in this field of research (Mittal et al., 1996; Russo et al., 2012). Different mechanisms of action have been described for GAPs:

Ras, Rho, Rac and Rab GTPases contain a glutamine in the switch II region (*e.g.* Ras Gln61, Rho Gln63) that is responsible for correctly placing the catalytic water molecule for a nucleophilic attack on the phosphate. In this case the GAP usually provides an arginine (arginine finger) to stabilize this glutamine during hydrolysis (Fig. 2A) (Scheffzek and Ahmadian, 2005). Interestingly, mutations in the P-loop of these GTPases (e.g. Gly12 or Gly13 in Ras) block the access to the respective arginine and thereby render GTPases hyperactive (Cherfils and Zeghouf, 2013). Arf GAPs also seem to function similarly, but in concert with coatamer that could also supply a catalytic arginine (Fig.2C) (Goldberg, 1999). The arginine can also be

supplied in *cis* as is the case with the α-subunit of the heterotrimeric G protein where the Regulator of G protein Signaling (RGS) GAP family member accelerates GTP hydrolysis by stabilizing the switch regions (Fig. 2B) (Scheffzek and Ahmadian, 2005).

Rap GTPases have a totally different mechanism: a threonine residue instead of a glutamine is present in the switch II region. No catalytic arginine has been identified in Rap GAPs, but an asparagine is crucial and has been proposed to play in trans a role that is analogous to the water positioning glutamine in Ras switch II (Fig. 2D). A tyrosine (Rap2 Tyr32) stabilizing the transition state is provided in cis by the switch I region and thus play a similar role to the catalytic arginine provided by Ras-GAP (Cherfils and Zeghouf, 2013).

Ran GTPases contain all the machinery for GTP hydrolysis. Catalytic residues are provided in cis and, similarly to Rap GTPases, a tyrosine in the switch I region stabilize the transition state. Hence, Ran GAPs mainly act in the stabilization of switch regions and don't provide catalytic residues (Cherfils and Zeghouf, 2013) (Fig.2E).

Finally, a unique mechanism to increase the GTP hydrolysis rate was found in the GAD GTPase family of proteins where dimerization takes place between two GTPases. Each partner provides critical residues to accelerate hydrolysis and also exchange of nucleotides on the other GTPase, rendering this family independent from any external GAP or GEF (Fig. 2F) (Gasper et al., 2009).

GAPs can be promiscuous and act on more than one GTPase. As a consequence, tight regulation is required to confer specificity in their activity. So far, it seems that GAP domain activity is not regulated by conformational changes. Instead, the timing of expression and degradation, as well as the spatial distribution seem to be important elements in GAP regulation. Many GAPs have domains that interact with lipids and membranes, and in some cases it has been shown that phospholipids or diaclygylcerol can regulate their localization (Bigay et al., 2003) (Canagarajah et al., 2004).

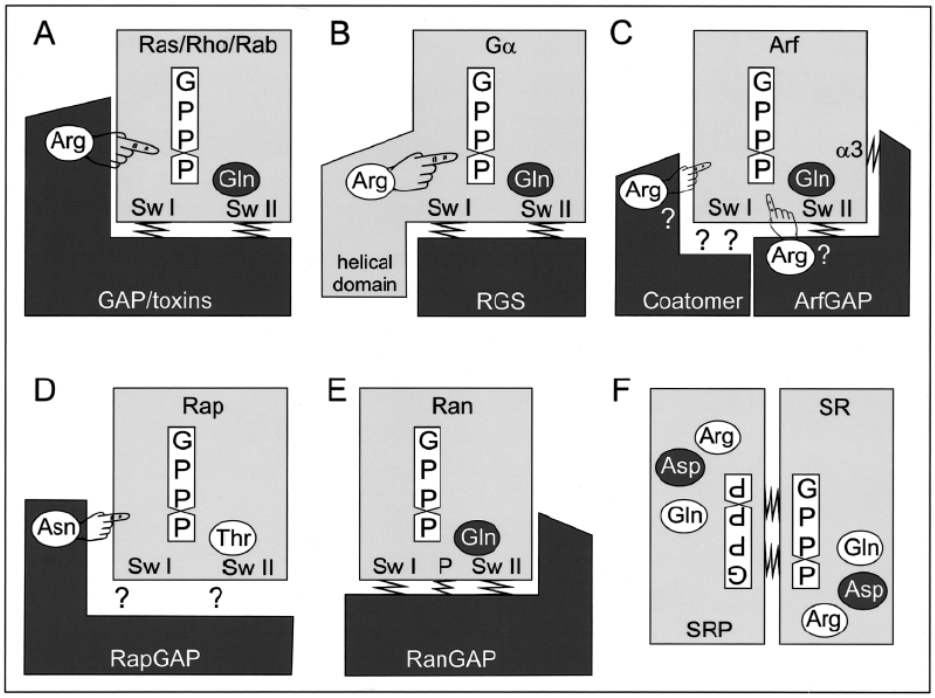

Fig. 2 The different GAP mechanisms. GTPases are represented in grey with important residues highlighted. SwI, SwII and P stand for Switch I, Switch II and P-loop, respectively. GAPs are represented in dark grey with a finger representing the critical catalytic residues. Taken from (Scheffzek and Ahmadian, 2005).

e) Guanine nucleotide Exchange Factors

GTPases bind very tightly to GTP or GDP and need external help to release GDP after hydrolysis in order to be reactivated with GTP. The catalytic domains of GEFs can be very different from one to the other. However all GEFs use the same mechanism to promote nucleotide exchange: they deform the switch I/II region to decrease GTPase affinity for the nucleotide (Bos et al., 2007). Destabilizing the binding of the Mg^{2+} ion in the GTPase nucleotide-binding site is commonly used by GEFs to release the nucleotide. Typically, the exchange reaction starts by a low affinity binding of the GEF to the GTPase bound to GDP. Once the GDP is released, the affinity of the GEF for the nucleotide-free GTPase is strongly increased (this particularity is considered a hallmark of GEFs). Finally a GTP is bound, which strongly decreases or even abolishes the GTPase-GEF interaction (Bos et al., 2007). GTPases have a similar affinity for GTP and GDP, but since GTP levels are usually 10 higher than GDP levels within cells, there is high probability that any GTP/GDP-free GTPase will be quickly reloaded with a new GTP (Bos et al., 2007).

f) Guanine Dissociation Inhibitors

A third class of regulatory proteins is the Guanine Dissociation Inhibitors (GDI). This name was given based on the original discovery that RhoGDI is an inhibitor of guanine dissociation. Today, however, it is known that the main function of GDIs is to relocalize lipid-modified GTPases and maintain them in a soluble inactive state (Dirac-Svejstrup et al., 1997). In fact many GTPases are attached to membranes via prenylation, farnesylation or geranylgeranylation. GDIs are able to extract these GTPases from the membranes and solubilize them by shielding their highly hydrophobic region, thus sequestering them from their effector proteins (Cherfils and Zeghouf, 2013).

Nutrient sensing

Eukaryotic cells have developed signaling pathways to adapt their growth in response to the availability of nutrients and/or, as in higher eukaryotes, to the presence of growth factors. The budding yeast *Saccharomyces cerevisiae* is a model organism where nutrient sensing has been intensely studied and pathways involved in transmitting information on essential building blocks for life (*i.e.* carbon, phosphate, sulfur and nitrogen) have been described in great detail. Core components of many of these pathways tend to be conserved among eukaryotes, underscoring their general importance in living cells. How yeast senses carbon, phosphate, sulfur and nitrogen sources, has been extensively reviewed (Conrad et al., 2014; De Virgilio, 2011; Smets et al., 2010; Zaman et al., 2008) and will not be further discussed here. Instead, I will specifically focus on a central hub that regulates cell growth in response to nutrients: the TOR pathway.

The TOR pathway

In 1975, a group of researchers isolated a soil bacterium (*i.e. Streptomyces hygroscopius)* from soil of the island Rapa Nui (Easter island). This bacterium was later found to produce a compound with antifungal properties coined rapamycin (Sehgal et al., 1975; Vézina et al., 1975). Further characterizations demonstrated that the compound was a macrocyclic lactone with immunosuppressive and anti-tumor effects in mammalian cells (Houchens et al., 1983). It was only in 1991, however,

when the mode of action of rapamycin was elucidated in yeast and that the Target Of Rapamycin (TOR) was identified. Rapamycin forms a complex with the proline isomerase Fpr1 (ortholog of FKBP12 in human) and then targets the highly conserved Ser/Thr TOR kinases (of which *S. cerevisiae* possesses two paralogs Tor1 and Tor2) (Heitman et al., 1991). Tor1 and Tor2 are 67% identical (and 82% similar) to each other and are members of the Phosphatidyl-Inositol Kinase-related Kinase (PIKK) family. All eukaryotic genomes sequenced so far harbour a *TOR* homolog, but unlike yeast, which has two *TOR* genes, most eukaryotes have only one copy (called mTOR in mammals for mammalian or mechanistic TOR) (Keith and Schreiber, 1995). In budding yeast deletion of *TOR2* is lethal causing random cell cycle arrest, while this is not the case for *TOR1* deletion. Cells deleted for both *TOR1* and *TOR2* are also unable to grow, but arrest in a G_0-like quiescent state, indicating that *TOR1* and *TOR2* share a redundant role in G_1-S progression and that *TOR2* may have an additional function that *TOR1* cannot provide (De Virgilio and Loewith, 2006a).

TOR is a multidomain protein containing approximately 30 tandem HEAT (Huntingtin, Elongation factor 3, A subunit of PP2A, and TOR) repeats at its amino terminus. HEAT repeats are composed of 40-50 residues forming α-helices that mediate protein-protein interactions (Andrade and Bork, 1995; Andrade et al., 2001). They cover about half of the TOR protein(s) and recruit important TOR partners such as Tco89 or Kog1 (human Raptor). The other half of the TOR protein contains a 600 residues-long FAT (Focal Adhesion Targeting) domain that is, together with the carboxy-terminal FATC domain, a typical feature of proteins of the PIKK family (Fig.3). Between the FAT and FATC domains, there is the 100-residues long FRB (FKBP12-Rapamycin-Binding) domain followed by the kinase domain and a 40-residues long region responsible for Lst8 binding called LBE. A recent structural study demonstrated that even if the kinase domain is 300 residues larger than most other kinases, it adopts a classical bi-lobal conformation with FRB forming the N-lobe and FATC, together with LBE, the C-lobe (Yang et al., 2013). The FAT domain is mainly composed of α-helices and wraps the kinase domain in a croissant shape. Unlike other kinases, the TOR kinase domain seems to be in a constitutively active conformation. It has been proposed that the FRB domain acts as a gatekeeper and regulates substrate access to the kinase domain (Yang et al., 2013). The position of the FRB suggests that the FKBP12-rapamycin complex inhibits TOR by blocking the access of substrates to the kinase domain (Alessi and Kulathu, 2013).

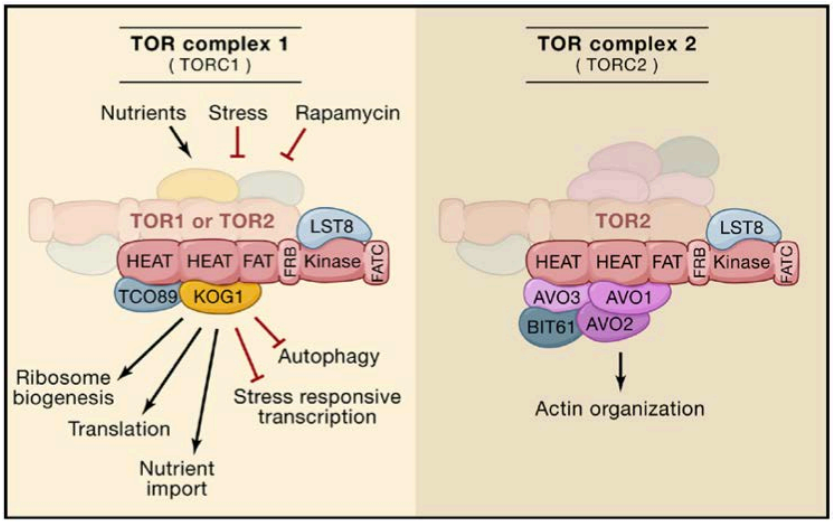

Fig. 3 Composition of TORC1 and TORC2 complexes. TOR is a multi-domain protein that can be found in two distinct complexes. The two complexes carry out different functions as indicated by black arrows (processes activated by the complex) and red bars (processes inhibited by the complex). Taken from (Wullschleger et al., 2006).

TOR can be found in two structurally and functionally distinct multiprotein complexes coined TOR complex 1 (TORC1) and 2 (TORC2), with only TORC1 being sensitive to rapamycin (Loewith et al., 2002). In yeast, a dimer of Tor1 and/or Tor2 forms the core component of TORC1. Lst8, Kog1 and Tco89 are additional subunits of the complex (Fig.3) (Wullschleger et al., 2006). The function of these latter three proteins is not clear but they might be involved in substrate binding, localization or in the reception of upstream signals. The complex localizes at the membrane of the vacuole: the major nutrient reservoir in yeast cells (Aronova et al., 2007; Cardenas and Heitman, 1995; Sturgill et al., 2008). Whereas Tco89 seems to be yeast specific, the mammalian ortholog of Kog1, Raptor, as well as mLst8, bind to the mTOR dimer. Additionally two non-conserved subunits have been identified: the negative regulators PRAS40 and DEPTOR (Tab.1) (Peterson et al., 2009; Sancak et al., 2007). Cryo-electron microscopy provided an initial insight into the three-dimensional structure of TORC1, confirming its dimeric organization. Intriguingly, the complex harbors a central cavity that the authors speculate could allow substrates with multiphosphorylation sites to shuffle between the two TOR catalytic domains inside the dimer (Yip et al., 2010). Another study found that Kog1 WD40 repeats interact with TOR N-terminal HEAT repeats, placing the Kog1 N-terminal close to the TOR kinase domain (Adami et al., 2007). Together with recent crystallographic data showing that Lst8 is also located close to the kinase domain (Yang et al., 2013), it

can be envisioned that Kog1 and Lst8 regulate access to the catalytic site and fine-tune substrate specificity. However, further investigations are needed to understand how TORC1 differentiates between the quality of its substrates and phosphorylates them accordingly (Kang et al., 2013).

Lst8 is also found in TORC2, which possesses a core dimer composed exclusively of Tor2. Avo1, Avo2 and Avo3 as well as Bit61 and its paralog Bit2 complete the composition of TORC2 (Fig.3) (Wullschleger et al., 2006). Like for the TORC1 components, the function of TORC2 subunits is far from being understood. However TORC2 specific components should hinder the access of Fpr1-rapamycin complex to the FRB domain, explaining why only TORC1 is sensitive to rapamycin treatment (Loewith et al., 2002). TORC2 localizes in discrete dots at the plasma membrane (Cardenas and Heitman, 1995; Kunz et al., 2000; Sturgill et al., 2008). In mammals, mTORC2 is composed of an mTOR dimer, mLst8, Avo1 and Avo3 orthologs named mSin1 and Rictor, respectively, Protor1 and 2 that are Bit61/Bit2 orthologs, and the mammalian specific inhibitor DEPTOR (Tab.1) (Cybulski and Hall, 2009).

TORC1		**TORC2**	
S. cerevisiae	***H. sapiens***	***S. cerevisiae***	***H. sapiens***
Tor1/Tor2	mTOR	Tor2	mTOR
Lst8	mLst8	Lst8	mLst8
Kog1	Raptor	Avo3	Rictor
-	PRAS40	Avo2	-
Tco89	-	Avo1	mSin1
-	DEPTOR	-	DEPTOR
		Bit61/Bit2	Protor1/2

Table 1 Components of TORC1 and TORC2 in *S. cerevisiae* and their counterparts in humans.

Interestingly, rapamycin treatment mimics many effects of nutrient starvation or exposure to stress. These include specifically, cell cycle arrest and entry into G_0, downregulation of general protein synthesis, accumulation of reserve carbohydrate such as trehalose, upregulation of stress response genes and autophagy, and alterations in nitrogen and carbon metabolism (Smets et al., 2010). Thus, TORC1 promotes anabolic processes and represses catabolic processes in response to nutrient quality and availability. The unavailability of a specific drug inhibiting TORC2

has delayed our understanding of its downstream regulation when compared to TORC1. Nevertheless TORC2 has been shown to regulate actin organization and plasma membrane homeostasis (Berchtold et al., 2012; Cybulski and Hall, 2009). Thus, it appears that TORC1 regulates the temporal aspects of cell growth, whereas TORC2 modulates spatial aspects of growth. Here I will focus on the processes regulated by TORC1 and on its upstream regulators.

TORC1 effectors

Many proteins involved in a wide range of cellular process are regulated in a TORC1 dependent manner. However, in the majority of cases, the precise mechanisms behind this regulation are not well understood. So far, two main effectors propagating TORC1 signals have been identified: Sch9 and the PP2A (and PP2-related) phosphatase (Huber et al., 2009).

i) Sch9

Sch9 is a non-essential kinase from the PKA, PKG, PKC (AGC) protein kinase family (Pearce et al., 2010). TORC1 regulates its activity via the phosphorylation of six serine and threonine residues located in the C-terminus of the protein (Fig.4) (Urban et al., 2007). Additionally, phosphorylation of threonine 570 by Pkh1 and Pkh2 is required for full Sch9 activation (Urban et al., 2007). Although Sch9 shares homology with mammalian Akt/PKB (Bergsma and Thevelein, 2000), its mammalian functional counterpart is the S6 kinase (S6K) that is also a well-known mTORC1 target (Urban et al., 2007).

ii) TAP42-phosphatase complex

The PP2A holoenzyme is composed of one of the two redundant catalytic subunits Pph21 or Pph22, a scaffolding subunit Tpd3, and a regulatory subunit Cdc55 or Rts1 (Jiang, 2006). The PP2A-related phosphatase is composed of the Sit4 catalytic subunit, and one of the four regulatory subunits: Sap4, Sap155, Sap185 or Sap190 (Jiang, 2006). TORC1 impinges on phosphatases by phosphorylation of Tap42 and Tip41 (Fig.4). Tap42 associates with the phosphatase catalytic subunit in a Tap42-Rrd2-Pph21/22 or Tap42-Rrd1-Sit4 complex (Düvel and Broach, 2004). Under

favorable growth conditions, these complexes reside mainly at membranes. However upon rapamycin treatment or nitrogen starvation, these complexes leave the membranes for the cytosol where Tap42 becomes dephosphorylated and subsequently releases the catalytic subunits (Zheng and Jiang, 2005). Tip41 recognizes and binds dephosphorylated Tap42, accelerating the Rrd2-Pph21/22 or Rrd1-Sit4 release (Jacinto et al., 2001). Tap42 has been described as a negative regulator of phosphatases in some cases and as an activator in others (Cherkasova and Hinnebusch, 2003; Düvel et al., 2003; Van Hoof et al., 2001). Current models rather describe Tap42 and Tip41 as modulators of PP2Ac and Sit4 substrate specificity in response to TORC1 activity (Jiang, 2006).

Processes regulated by TORC1

Here I will review the processes through which TORC1 promotes the accumulation of mass and inhibits both the stress response as well as entry into the quiescence state during cell growth phases.

i) Protein Synthesis

TORC1 regulates protein synthesis by impinging on ribosome biogenesis, translation and mRNA stability.

a) Ribosome biogenesis

TORC1 positively regulates the expression of rRNAs, Ribosomal Proteins (RPs), and the Ribosome Biogenesis (RiBi) regulon (Jorgensen et al., 2004). TORC1 promotes the interaction between Rrn3 and RNA polymerase I, which is necessary for the recruitment of the polymerase to the 35S rDNA promoter and transcription of the 5.8S, 18S and 25S precursor 35S rRNA (Claypool et al., 2004). Hmo1, which is required for transcription of the 35S rDNA, is also stabilized by TORC1 (Fig.4) (Berger et al., 2007). Maf1, a RNA polymerase III repressor, is directly (or indirectly via Sch9) inhibited by TORC1, thus promoting expression of 5S rRNA and tRNA (Fig.4) (Huber et al., 2009; Oficjalska-Pham et al., 2006; Wei et al., 2009). RP gene expression is promoted by TORC1 via stabilization of the Ifh1-Fhl1 complex (Martin et al., 2004; Schawalder et al., 2004; Wade et al., 2004) and recruitment of the NuA4

histone acetyltransferase to RP promoters (Reid et al., 2000; Rohde and Cardenas, 2003). Sfp1 relocalizes to the nucleus after TORC1 phosphorylation and subsequently activates the expression of RP and RiBi genes (Fig.4) (Lempiäinen et al., 2009; Marion et al., 2004). TORC1 also inhibits transcriptional repressors of RiBi genes, such as Stb3 and Dod6/Tod6, *via* Sch9 (Fig.4) (Huber et al., 2009; Liko et al., 2010; 2007). Finally TORC1 promotes ribosome assembly by preventing nuclear entrapment of the 40S synthesis factors Dim2 and Rrp12 (Vanrobays et al., 2008).

b) Translation

Active TORC1 promotes translation on one hand by indirectly controlling the phosphorylation status of the α-subunit of the eukaryotic initiation factor 2 (eIF2α) through Sch9 (Urban et al., 2007), and on the other hand by maintaining the eIF2α kinase Gcn2 inactive, via inhibition of Sit4-mediated dephosphorylation of its serine 577 (Fig.4) (Cherkasova and Hinnebusch, 2003). In other words, TORC1 indirectly prevents the inhibitory phosphorylation of eIF2α and consequently promotes translation initiation. It has also been proposed that TORC1 controls translation initiation via eIF4E, possibly by eIF4G stabilization and Eap1 negative regulation (Barbet et al., 1996) (Berset et al., 1998; Cosentino et al., 2000; Danaie et al., 1999; Kuruvilla et al., 2001). Under stress conditions such as nutrient limitation, cells repress general translation but activate a specific program to express genes required for entry into the quiescent state G_0 (Radonjic et al., 2005). The PAS kinase Rim15 is essential for this transcriptional reprogramming (Pedruzzi et al., 2003). Rim15 is homologous to the human greatwall kinase and is negatively regulated by both PKA and TORC1. In cells growing exponentially, the TORC1 effector Sch9 directly phosphorylates Rim15, promoting its cytoplasmic retention by association with the 14-3-3 protein Bmh2 (Wanke et al., 2008). After TORC1 inhibition, active Rim15 shuttles to the nucleus, and positively acts on the general stress transcription factors Msn2, Msn4 and the post diauxic-shift transcription factor Gis1 (Fig.4) (Cameroni et al., 2004). The precise mechanism by which Rim15 activates Msn2/4 dependent transcription is not known. However a recent study showed that Rim15 promotes Gis1 activity via the yeast endosulfines Igo1 and Igo2. Rim15 directly phosphorylates Igo1/2, which subsequently inhibit the $PP2A^{Cdc55}$ phosphatase, thereby preventing dephosphorylation and consequently favoring promoter recruitment of Gis1 (Bontron et al., 2013).

c) mRNA stability

As previously discussed, after nutrient deprivation, ribosome biogenesis and translation initiation are decreased, which results in downregulation of general protein production in order to save energy. Most mRNAs are also sent for degradation. Nevertheless, the expression of a specific subset of genes, and protection of the respective mRNAs, are required for the proper response to starvation (Radonjic et al., 2005). Upon TORC1 inactivation, the Rim15 targets Igo1 and Igo2 bind a specific set of mRNAs, to protect (indirectly, likely via inhibition of $PP2A^{Cdc55}$; see above) them from decapping and subsequent 5'-3' degradation (Fig.4) (Luo et al., 2011; Talarek et al., 2010).

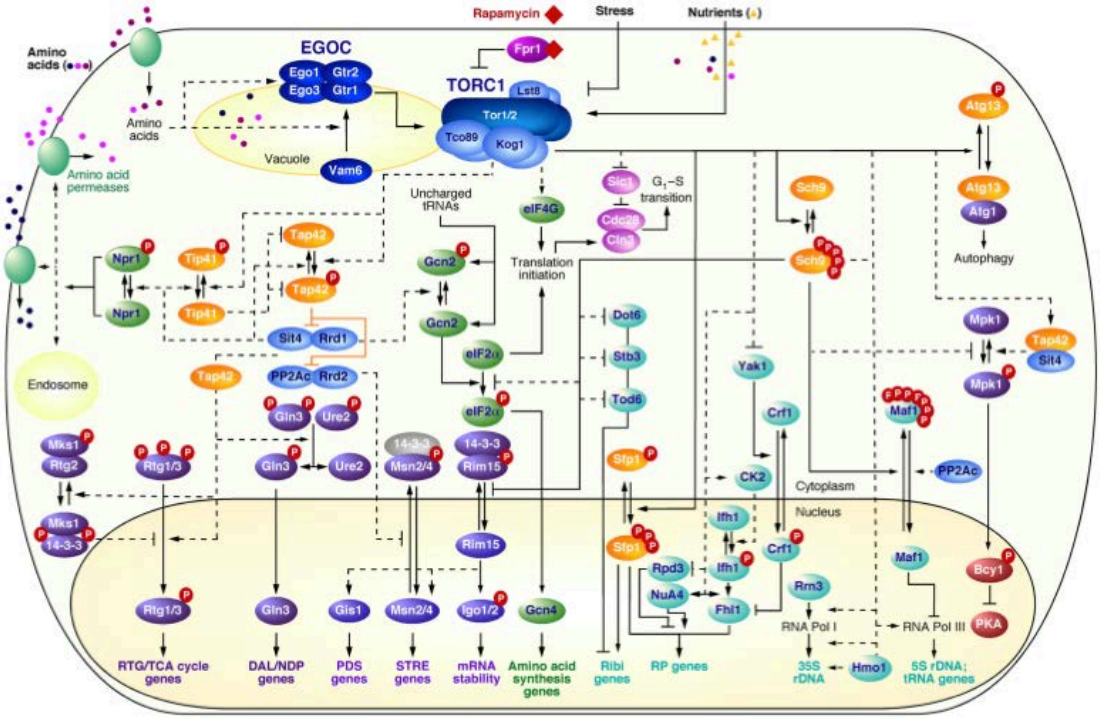

Fig. 4 The TORC1 signaling network in *S. cerevisiae*. Proximal TORC1 effectors are in orange. Anabolic processes that are positively regulated by TORC1 are in green (*i.e.* translation initiation and permease activity) or in turquoise (*i.e.* expression of translation machinery), whereas catabolic processes repressed by TORC1 are in purple (*i.e.* autophagy, transcriptional stress response). Upstream regulators are in dark blue. Red balls containing a P denote phosphorylation. Arrows show activation events and bars indicate inhibition. Dashed lines stand for indirect and/or potential interactions, whereas solid lines denote direct interactions. See text for more details. Taken from (De Virgilio, 2011).

ii) TORC1 and amino acid homeostasis

Budding yeast are able to synthesize all 20 amino acids that are used for protein production. Nevertheless, yeast preferentially import amino acids when they are present in sufficient amounts in the environment. Interestingly, amino acids, especially branch-chained amino acids such as leucine, isoleucine and valine, represent the most potent TORC1 stimulators and cannot be compensated by any other stimulus (Binda et al., 2009; Crespo et al., 2002; Xu et al., 1998). In turn, TORC1 feeds back on processes that control amino acid homeostasis at different levels. In this section I will discuss the mechanisms that allow the cell to gauge external and internal amino acid levels and how TORC1 activity impinges on them.

a) Amino acid permeases

Depending on the quality of the nitrogen source, yeast cells express and target to the plasma membrane specific sets of Amino Acid Permeases (AAP). Under nutrient rich conditions, high-affinity and high-specificity AAPs are sorted to the membrane (*e.g.* the tryptophane permease Tat2). After amino acid depletion, these transporters are internalized and sent for degradation, and several broad-specificity and high-capacity transporters are expressed and replace them at the plasma membrane (*e.g.* the general amino acid permease Gap1 and the proline permease Put4) (Ljungdahl and Daignan-Fornier, 2012). TORC1 activity influences the turnover of several permeases by promoting phosphorylation of the kinase Npr1 through control of the Tap42-Sit4 phosphatase complex (Fig.4) (Schmidt et al., 1998). After rapamycin treatment or nitrogen limitation, Npr1 is dephosphorylated, becomes activated and indirectly promotes Tat2 ubiquitination that triggers its internalization to vacuolar membrane (Beck et al., 1999). In contrast Gap1 is stabilized at plasma membrane via a mechanism involving Npr1 and two arrestin-like proteins Bul1 and Bul2. Active Npr1 phosphorylates Bul1/2, which promotes their interaction with the 14-3-3 proteins Bmh1/2, maintaining them inactive. Upon switching to a good nitrogen source, Bul1/2 are dephosphorylated in a Sit4-dependent manner and released from the 14-3-3 proteins (Merhi and André, 2012). This allows the Rsp5 ubiquitin ligase to form a complex with Bul1/2, which targets Gap1. Ubiquitinated Gap1 is then internalized (Yashiroda et al., 1998; 1996). A similar mechanism takes place for the regulation of the arginine permease Can1: when TORC1 is active, the arrestin Art1 in complex

with Rsp5 localizes at the plasma membrane where it promotes Can1 ubiquitination and endocytosis. Upon TORC1 inhibition, active Npr1 phosphorylates the N-terminus of Art1. This inhibits the Art1-Rsp5 complex and results in its relocalization to the Golgi and cytoplasm. As a consequence, Can1 is stabilized at the plasma membrane (MacGurn et al., 2011). TORC1 also regulates the plasma membrane ammonium transporter Mep2. In contrast to Tat2 or Gap1, TORC1 does not affect Mep2 membrane sorting but instead influences its activity. When TORC1 is downregulated, Npr1 directly phosphorylates Mep2 and promotes its transport activity. Upon glutamine addition, TORC1 is reactivated and inhibits Npr1, allowing two plasma membrane phosphatases, Psr1 and Psr2, to dephosphorylate and inactivate Mep2 (Boeckstaens et al., 2014).

b) Autophagy

Autophagy is a process conserved among eukaryotes in which elements of the cytoplasm such as long-lived proteins, protein aggregates, organelles or even invading pathogens are targeted to the vacuole (or lysosome in mammals) for degradation and recycling of their components (Devenish and Klionsky, 2012). Two types of autophagy have been described: microautophagy and macroautophagy. In microautophagy, cytoplasmic material is directly trapped by invagination of the vacuolar membrane. Macroautophagy is initiated by the Pre-Autophagosomal Structure (PAS) that forms a double lipid bilayer around cytoplasmic material, eventually resulting in the formation of a mature autophagosome. Fusion of the autophagosome with the vacuolar membrane results in the delivery of a single membrane vesicle inside the vacuolar lumen. In both microautophagy and macroautophagy, the vesicles delivered inside the vacuolar lumen and their contents are degraded by hydrolases (Levine and Klionsky, 2004). There is a constant basal level of autophagy inside cells, which is strongly increased during nutrient starvation or cellular stress (Nakatogawa et al., 2009). Under starvation conditions, the induced-autophagy serves to provide building blocks to maintain essential metabolism for adaptation and survival. Apart from stress conditions, autophagy is necessary to remove deleterious elements from the cell such as misfolded proteins, protein aggregates, old organelles, or pathogens. Selective autophagy targets these deleterious elements with the help of adaptor proteins (Devenish and Klionsky, 2012). Specific names are given to these processes depending on their target:

aggrephagy for protein aggregates (Webb et al., 2003), mitophagy for mitochondria (Kissová et al., 2004), pexophagy for peroxisomes (Sakai et al., 1998) ribophagy for ribosomes (Kraft et al., 2008) and xenophagy for pathogens (Dupont et al., 2010). In *S. cerevisiae*, 35 AuTophaGy-regulated (ATG) genes have been identified so far, with 15 of them coding for the core machinery commonly needed for membrane formation (Devenish and Klionsky, 2012). Among them, Atg13 plays a central role in PAS formation. Atg13 binds and upregulates the Atg1 kinase and promotes recruitment of other Atg proteins required for autophagosome formation. Under nutrient rich conditions, TORC1 and PKA pathways tightly repress autophagy. TORC1 maintains Atg13 in its hyperphosphorylated form that prevents its interaction with Atg1 (Fig.4). After Rapamycin treatment Atg13 is rapidly dephosphorylated and autophagy is induced (Kamada et al., 2000; 2010). It is possible that PP2A mediates Atg13 dephosphorylation after TORC1 inactivation, as it has been shown that the Tap42-PP2A module negatively regulates autophagy (Yorimitsu et al., 2009). PKA mediates phosphorylation of both Atg1 and Atg13, preventing their interactions with other PAS components (Stephan et al., 2009). Amino acids recycled from autophagy-degraded proteins are exported from the vacuole to the cytoplasm through the membrane effluxer Atg22 and additional amino acid vacuolar transporters (Yang et al., 2006). Upon prolonged starvation, exported amino acids are then able to reactivate TORC1 and attenuate autophagy (Shin and Huh, 2011).

c) The nitrogen catabolite repression pathway

Yeast cells are able to discriminate between good nitrogen sources that can easily be converted into amino acid precursors, and less good sources that need more energy to be metabolized. The Nitrogen Catabolite Repression (NCR) pathway ensures that in the presence of a preferred nitrogen source (i.e. ammonia, glutamate and glutamine), expression of genes required for the use of poor nitrogen sources (i.e. proline, urea, allantoin and GABA) is repressed (Conrad et al., 2014). Four GATA family zinc-finger transcription factors are involved in the expression of NCR target genes: two repressors, Dal80 and Gzf3, and two activators, Gln3 and Gat1 (Coffman et al., 1995; 1996; Cooper, 2002; Minehart and Magasanik, 1991). The phosphorylation status of Gln3 and Gat1 dictate their localization. In the presence of rich nitrogen sources, Gln3 and Gat1 are sequestered in the cytoplasm where Ure2 acts as an anchor for Gln3 (Magasanik and Kaiser, 2002). Upon rapamycin treatment

or nitrogen starvation, Gln3 relocalizes to the nucleus where it activates its transcriptional program. TORC1 inhibition of the Tap42-Sit4 phosphatase complex promotes Gln3 phosphorylation, thus maintaining its interaction with Ure2 and hence its cytoplasmic localization (Fig.4) (Beck and Hall, 1999). An additional level of TORC1 control on Gln3 occurs via the promotion of Ure2 phosphorylation via an unknown mechanism (Cardenas et al., 1999; Hardwick et al., 1999). Gat1 relocalization to the nucleus is also triggered by rapamycin treatment. The mechanism remains elusive, but seems to be different from the control of Gln3 as it is independent from Ure2 or Sit4 (Georis et al., 2008; Kuruvilla et al., 2001). A recent study proposed that in parallel to TORC1, the NCR pathway integrates inputs from multiple pathways (Tate and Cooper, 2013). Among the genes controlled by the NCR pathway there are permeases required for growth on poor nitrogen sources such as the broad-specificity and high-capacity general amino acid permease Gap1 (André et al., 1993; Jauniaux and Grenson, 1990; Van Zeebroeck et al., 2009) and the ammonium permeases Mep1,Mep2, and Mep3 (Marini et al., 1994) (Marini et al., 1997).

d) The retrograde response pathway

Under fermentation conditions, the TriCarboxylic Acid (TCA) cycle is repressed. However, if yeast cells under these conditions do not have access to a good nitrogen source, they must maintain the first steps of the TCA cycle in order to produce α-ketoglutarate, which can subsequently be converted to glutamate and then to glutamine via the glutamate deshydrogenase Gdh1 and the glutamine synthase Gln1, respectively (Liu and Butow, 2006). While Gdh1 and Gln1 expression is dependent on Gln3, the expression of the TCA enzymes responsible for α-ketoglutarate production from oxaloacetate is controlled by the ReTroGrade response pathway (RTG) (Conrad et al., 2014). Under nutrient rich conditions, two transcription activators, Rtg1 and Rtg3, are retained in the cytosol by phosphorylated Mks1 and the 14-3-3 proteins Bmh1/2 (Dilova et al., 2004). Following glutamine or glutamate depletion, Mks1 is dephosphorylated allowing the positive regulator Rtg2 to compete for Bmh1/2 binding, resulting in the release of the Rtg1-3 dimer (Dilova et al., 2004). Rtg1-3 is then able to enter the nucleus and activate transcription of RTG genes (Fig.4) (Butow and Avadhani, 2004). Dephosphorylated Mks1 is recognized by Grr1, ubiquitinated and sent for degradation (Zaman et al., 2008). TORC1 negatively

regulates the RTG pathway via Lst8 (Chen and Kaiser, 2003). Nevertheless this regulation seems to be rather indirect, as TORC1 does not participate in nutrient regulation of this pathway (Giannattasio et al., 2005) and may be a consequence of altered nitrogen metabolism (Tate and Cooper, 2003).

e) The general amino acid control

TORC1 represses the General Amino Acid Control (GAAC) pathway, a signaling node able to activate genes needed for amino acid and purine synthesis in response to amino acid starvation (Hinnebusch, 2005). The pathway is activated by uncharged tRNAs that directly bind to the Gcn2 proetin kinase (Dong et al., 2000; Wek et al., 1989), provoking Gcn2 autophosphorylation and subsequent eIF2α phosphorylation (Qiu et al., 2002; Romano et al., 1998). This results in a general inhibition of translation initiation, but the specific activation of *GCN4* mRNA translation (Dever et al., 1992). Gcn4 is the transcription factor responsible for initiating the GAAC transcription program that targets more than 500 genes (Mösch et al., 1991; Natarajan et al., 2001). Phosphorylation of Gcn2 on serine 577 has an inhibitory effect on Gcn2 activity and prevents Gcn4 translation. TORC1 promotes Gcn2 S577 phosphorylation indirectly by maintaining Sit4-Tap42 phosphatase inactive (Fig.4) (Cherkasova and Hinnebusch, 2003; Rohde et al., 2004; Valenzuela et al., 2001). Thus, TORC1 positively regulates translation initiation and inhibits Gcn4 synthesis.

f) Sensing of extracellular amino acids by the SPS pathway

Named after its core components Ssy1, Ptr3 and Ssy5, the SPS pathway enables yeast to sense extracellular amino acids. Ssy1 is a plasma membrane protein from the Amino Acid Permease (AAP) family. However, no transport activity has been shown for Ssy1, and instead it functions as a sensor (Didion et al., 1998). Indeed, Ssy1 can be stimulated by different amino acids, with leucine being the most potent activator (Iraqui et al., 1999). External amino acid binding triggers a change in the conformation of Ssy1 to its signaling active form, whereas internal amino acid binding to Ssy1 promotes its signaling inhibitory conformation. As such, Ssy1 is able to sense the ratio between external and internal amino acids (Wu et al., 2006). Via its N-terminus, Ssy1 interacts with its signaling partners Ssy5 and Ptr3. Ssy5 is a serine endoprotease that can cleave itself into an inhibitory pro-domain and a catalytic

domain. Both domains remain non-covalently attached in a non-active complex. Upon amino acid stimulation, the Ssy5 pro-domain is phosphorylated by the yeast casein kinases Yck1 and Yck2, allowing recognition and ubiquitination by the SCF^{Grr1} ubiquitin ligase complex and subsequent degradation by the proteasome (Abdel-Sater et al., 2011). Ptr3 acts as an adaptor mediating Yck1/2 recruitment to Ssy1 (Omnus and Ljungdahl, 2013). Ssy5 pro-domain phosphorylation can be removed by the $PP2A^{Rts1}$ phosphatase complex (Omnus and Ljungdahl, 2013). Once released from the pro-domain, activated Ssy5 catalytic domain targets Stp1 and Stp2, two partially redundant transcription factors (Fig.5). Stp1 and Stp2 are latent transcription factors retained in the cytoplasm via an N-terminal regulatory domain. Nevertheless, if any unprocessed Stp1/2 enter the nucleus, Asi inner nuclear membrane proteins (Asi1, Asi2, Asi3) will prevent their access to target genes (Boban et al., 2006; Zargari et al., 2007). The Ssy5 catalytic domain cleaves the Stp1/2 N-terminal domain inducing their nuclear translocation, where together with the transcriptional co-activator Dal81 they will induce transcription of SPS regulated genes such as the amino acid permease genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1*, *MUP1*, *TAT1*, *TAT2*, and the peptide transporter gene *PTR1* (Abdel-Sater et al., 2004; Andréasson and Ljungdahl, 2002; Didion et al., 1998; Klasson et al., 1999)

Some evidence indicates potential crosstalk between the SPS pathway and the TOR pathway. Like for the SPS pathway, the most potent amino acid activator of TORC1 is leucine (Binda et al., 2009). Interestingly, deletion of *STP1* renders cells hypersensitive to the TOR inhibitor rapamycin, whereas *STP1* overexpression confers rapamycin resistance, indicating that regulation of amino acid import via AAP and the SPS parthway could influence TORC1 activity (Shin et al., 2009). On the other hand, TORC1 can feedback on the SPS pathway: inactivation of TORC1 by rapamycin did not affect the SPS cascade, as Stp1 is processed normally, but resulted in the degradation of the nuclear cleaved Stp1 via the PP2A-like phosphatase Sit4 (Shin et al., 2009). This indicates that TORC1 may positively influence the duration of the SPS transcriptional response after external amino acid stimulation.

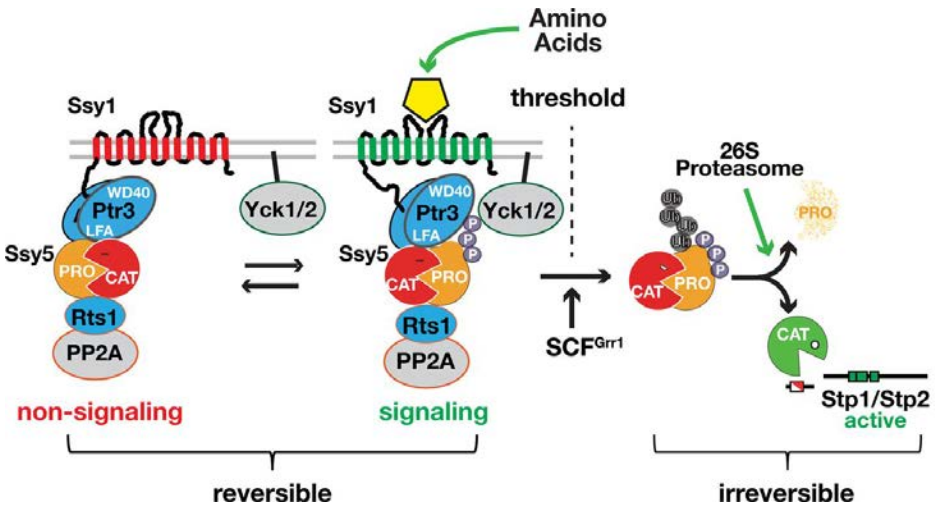

Fig.5 The SPS pathway. Regulation of the SPS pathway in the absence (left), or in the presence (right) of amino acids. See text for details. Taken from (Omnus and Ljungdahl, 2013).

TORC1 regulators

TORC1 is regulated by the abundance and/or the quality of nitrogen and carbon sources (De Virgilio and Loewith, 2006a; 2006b). Intracellular amino acids are able to activate TORC1, like treatment with cycloheximide, a translation elongation inhibitor that causes an increase in the intracellular free amino acid pool, causes rapid TORC1 activation (Binda et al., 2009; Urban et al., 2007). Amongst amino acids, glutamine and Branch Chained Amino Acids (BCAA), especially leucine, are the most potent activators (Binda et al., 2009; Crespo et al., 2002; Xu et al., 1998). TORC1 activity is rapidly shut down in response to stress like heat shock (Takahara and Maeda, 2012) or after treatment with direct inhibitors such as rapamycin or caffeine (Wanke et al., 2008). Finally, in higher eukaryotes, growth factors (*e.g.* insulin) are essential activators of TORC1.

Even if the cues regulating TORC1 are well known, the mechanisms through which they act remain obscure in the majority of cases. The branch allowing growth factors and stress to signal to mTORC1 in higher eukaryotes is the best characterized. Here I will briefly describe it.

i) The Rheb-TSC module

Rheb is a small GTPase that is able to directly bind and stimulate the kinase activity of mTORC1 when it is loaded with GTP (Saucedo et al., 2003). Rheb is negatively regulated by the trimeric TSC complex. Composed of Tsc1, Tsc2 and TBC1D7, the

TSC complex acts as a GAP and promotes the GDP-bound form of Rheb (Dibble et al., 2012; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003; Zhang et al., 2003). Insulin and Insulin-like growth factors promote TORC1 activity via activation of the PI3K pathway, leading to activation of Akt, which inhibits the TSC complex by directly phosphorylating Tsc2 (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Alternatively, growth factors phosphorylate Tsc2 via the Extracellular signal-Regulated Kinase ERK after activation of the Ras-Raf-MAPK/ERK kinase axis (Ma et al., 2005).

Crosstalk between mTORC1 and Wnt signaling occurs through the TSC complex: the Wnt signaling pathway restricts the activation of the TSC complex by Glycogen Synthase Kinase 3 β (GSK3β) and thus activates mTORC1 (Inoki et al., 2006).

The Rheb-TSC module is also required for stress sensing. Cellular energy level is directly linked to the level of intracellular ATP. Under stress conditions, ATP levels rapidly drop, while AMP levels increase. The AMP-activated Protein Kinase (AMPK) senses this increase in AMP and then activates the TSC complex (Hardie, 2007). Thus mTORC1 is inactivated when the cellular energy level is low. AMPK is also activated independently of ATP level in response to DNA damage via Sestrin 1 and 2, two transcriptional targets of p53 (Budanov and Karin, 2008). During hypoxia, mitochondrial functions are impaired leading to a decrease in the level of ATP that is detected by AMPK. However, hypoxia also regulates mTORC1 more directly via REDD1, a protein that promotes TSC complex assembly (Brugarolas et al., 2004).

In the fission yeast *Schizosaccharomyces pombe*, the Rheb-TSC module is present and regulates TORC1 (Urano et al., 2005; 2007; Uritani et al., 2006). Disruption of either *TSC1* or *TSC2* results in amino acid uptake deficiency, impaired gene induction upon nitrogen starvation, and sexual cycle defects (Matsumoto et al., 2002; van Slegtenhorst, 2003). So far, the cues that impinge on the Rheb-TSC module in fission yeast have not been clearly identified.

Saccharomyces cerevisiae does not have any Tsc1/2 homologs. A Rheb homolog, coined Rhb1, is present but so far has not been shown to regulate TORC1. Intriguingly, loss of Rhb1 increases arginine uptake and causes canavanine hypersensitivity in a process requiring the arginine-specific permease Can1 (Urano et al., 2000). A phenotype identical to that is observed in *S. pombe* cells lacking Rheb (Yang et al., 2001). Furthermore, Can1 sorting to the plasma membrane has been shown to be negatively regulated by TORC1 in *S. cerevisiae* (MacGurn et al., 2011). Finally, a yeast two-hybrid study showed that in budding yeast Rhb1 interacts with

the TORC1 subunit Tco89 and with the TORC1 upstream regulator Ego1 (Nicolas Panchaud, Master Thesis, 2009).

ii) The Rag GTPases module

Amino acids, especially BCAA such as leucine, are a major TORC1 input that cannot be compensated by any other stimulus (Efeyan et al., 2012b; Jewell et al., 2013). Until recently, the mechanism by which amino acids activate TORC1 was a complete mystery. Discovery of the highly conserved Rag GTPases Gtr1 and Gtr2 as part of the EGO complex (EGOC) gave an initial insight into this mechanism. Today accumulating evidence indicates that the EGOC is the central component of a primordial signaling branch regulating TORC1.

a) The Rag GTPases

The Ras-related GTP-binding protein (Rag) family of GTPases is particularly important to relay amino acid signals to TORC1. In higher eukaryotes, RagA or RagB forms a heterodimer with RagC or RagD (Sekiguchi et al., 2001). In *S. cerevisiae*, the RagA/B ortholog Gtr1 dimerizes with the RagC/D ortholog Gtr2 (Nakashima et al., 1999). Human RagA shares 90% identity in its sequence with RagB, but less than 25% with RagC and RagD, whereas RagC shares 87% sequence identity with RagD (Sekiguchi et al., 2001). Gtr1 shares 49% identity and 75% similarity with RagA, and Gtr2 shows 42% identity and 76% similarity with RagC (Gong et al., 2011). Rag GTPases are able to directly bind and stimulate TORC1 in response to amino acids (Binda et al., 2009; Sancak et al., 2008). A unique feature of Rag GTPases is that the dimer needs to be asymmetrically loaded to be fully active, *i.e.* when RagA,B or Gtr1 is loaded with GTP and RagC,D or Gtr2 loaded with GDP. Conversely, GDP-bound RagA,B or Gtr1, combined with GTP-bound RagC,D or Gtr2, is inactive (Binda et al., 2009; Gao and Kaiser, 2006; Kim et al., 2008; Sancak et al., 2008). In cells expressing constitutively active Rags (*i.e* RagA/B/Gtr1 GTP-locked and RagC/D/Gtr2 nucleotide-free forms), TORC1 is partially protected from amino acid deprivation, whereas cells missing Rags or expressing dominant negative Rag alleles are unable to stimulate TORC1 in response to amino acids (Binda et al., 2009; Kim et al., 2008; Sancak et al., 2008).

Rag GTPases differ from other members of the Ras GTPase family by their long carboxy-terminal domain (CTD), which does not contain any site for lipid modification and membrane anchoring (Bun-Ya et al., 1992). Recent crystallographic data show that Gtr1 and Gtr2 adopt a similar structure and interact via their extended CTDs to harbor a pseudo-twofold symmetry (Fig.6) (Gong et al., 2011). Dimerization through the CTDs is essential for Rags function (Gong et al., 2011; Sekiguchi et al., 2014). Both CTDs are very similar and together form a roadblock-like structure predicted to be rigid, thus ensuring a stable complex (Gong et al., 2011). In contrast, the N-terminal regions of Gtr1 and Gtr2, which form the G domain responsible for binding guanine nucleotides, are more divergent. Despite the fact that the G domain is very similar to the one of other small G proteins, the Gtr1 switch I region misses a tyrosine residue that would typically interact with the γ-phosphate of the GTP, stabilizing the transition state from GTP to GDP (Jeong et al., 2012). A similar feature is found in the Arf GTPases. Moreover, in contrast with most of the Ras family GTPases, the Gtr1 P-loop contains a serine (S15) that is able to interact with the γ-phosphate (Jeong et al., 2012). An equivalent serine in Rab3a has been shown to impose stereochemical constraints against GTP hydrolysis (Dumas et al., 1999). These two particularities explain the very low intrinsic hydrolysis rate of Gtr1 and highlight the importance of a GAP for its regulation (Sengottaiyan et al., 2012). Gtr2 has an atypical G domain. Its P-loop contains a conserved arginine able to stabilize the GTP to GDP transition state and to accelerate hydrolysis (Jeong et al., 2012). Furthermore, Gtr2 does not require Mg^{2+} to bind GDP. This results in a very low GDP binding affinity and rapid release of the nucleotide, raising the possibility that Gtr2 does not require any GEF (Sekiguchi et al., 2001). GTP hydrolysis triggers a large G domain rearrangement in both Gtr1 and Gtr2. When Gtr1 and Gtr2 are in a GTP-bound conformation, their G domain does not interact. However when Gtr2 is bound to GDP, part of its G domain comes into contact with the Gtr1 G domain (Jeong et al., 2012). This conformational change should not directly influence the hydrolysis rate of the GTPases as the interaction surface does not involve the guanine nucleotide-binding region, excluding direct intervention of residues in trans. However it cannot be excluded that the interaction between the G domains imposes steric constraints and that the conformation of one domain can influence the status of the other. It would be particularly interesting to determine if both Rags can harbor a GDP-bound conformation at the same time.

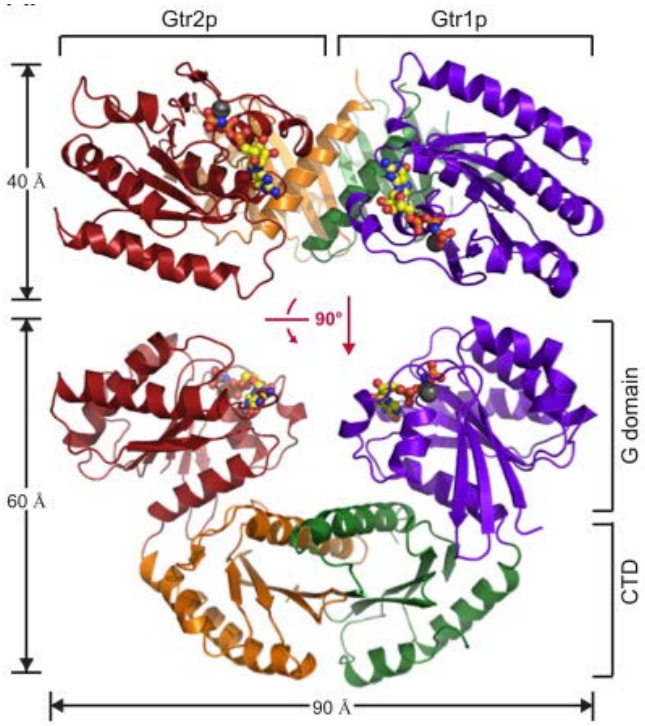

Fig. 6 Structure of Gtr1-Gtr2 heterodimer. Two different views of the structure with ribbon representation. The Gtr2 G-domain is in red and its C-terminal domain (CTD) in orange. The Gtr1 G-domain is in blue and its CTD in green. Both G-domains are bound to the GTP analog GMPPNP (represented as red, yellow and blue balls and sticks). A magnesium ion is represented as a grey ball. Taken from (Gong et al., 2011).

The major G domain rearrangements upon GTP hydrolysis modulate the interactions between Rags and their effectors. Amino acids promote the interaction between Rags and TORC1. GTP-bound Gtr1 more strongly interacts with the TORC1 subunits Tco89 and Kog1 than Gtr1-GDP (Binda et al., 2009). Gtr1-GTP interacts with the C-terminus of Tco89 and the central heat-repeat containing region of Kog1 (Sekiguchi et al., 2014). Similarly, the RagA/B nucleotide binding status also dictates its interaction with mTORC1 components, *i.e.* GTP-bound RagB binds tightly to the Kog1 ortholog Raptor (Sancak et al., 2008). The residues involved in this interaction are located close to the switch I/II and the P-loop: regions that exhibit substantial conformational rearrangements upon the hydrolysis of GTP to GDP (Gong et al., 2011). Furthermore, Rag heterodimers containing GDP-bound RagC bind more efficiently to mTOR and Raptor (Tsun et al., 2013).

Rags do not stimulate mTORC1 activity directly but instead promote its relocalization from the cytoplasm to the surface of the lysosome, where it meets the small GTPase Rheb (Sancak et al., 2008). In its GTP-bound form, Rheb directly stimulates mTORC1 kinase activity (Long et al., 2005). The mechanism by which the Gtr1-Gtr2

heterodimer activates TORC1 in budding yeast is not known and may differ from higher eukaryotes as there is currently no evidence indicating that the Rheb ortholog Rhb1 is involved in TORC1 signaling (Binda et al., 2010). Moreover TORC1 localizes to the vacuolar rim in both the presence and absence of leucine (Binda et al., 2009).

A recent study showed that in humans and flies the Rag GTPases are not only required to stimulate TORC1 activity, but also to properly inactivate it (Demetriades et al., 2014). Upon amino acid starvation, Rags switch to their inactive conformation (i.e. RagA/B-GDP, RagC/D-GTP). This has two consequences: i) It weakens the interaction between Rags and mTORC1, and ii) the inactive Rag dimer recruits the TSC complex from the cytoplasm to the lysosomal surface, where it can exert its GAP activity on Rheb. The switch from the GTP- to the GDP-bound conformation of Rheb allows complete mTORC1 release from the membrane and therefore a complete attenuation of mTORC1 signaling. This study brings interesting conceptual advances: i) mTORC1 localization is not only regulated by Rags but by a dual tethering mechanism involving Rheb and Rag GTPases. ii) Amino acids not only signal through the Rag module but also involve the Rheb-TSC module. This model also explains why in the past different studies had contradictory conclusions concerning the involvement of the Rheb-TSC module in amino acid signaling (Gao and Kaiser, 2006; Roccio et al., 2006; Smith et al., 2005).

Rags have been shown to primarily localize to the vacuolar/lysosomal membrane (Binda et al., 2009; Dubouloz et al., 2005; Gao et al., 2005; Sancak et al., 2008). However, it has also been reported that Gtr1 and Gtr2 localize in the nucleus and cytoplasm where they could have targets other than TORC1 (Nakashima et al., 1996; Sekiguchi et al., 2001). Indeed, it has been proposed that the Gtrs could influence nuclear transport through negative regulation of the Ran GTPase cycle (Nakashima et al., 1996; 1999). The Gtr1-Gtr2 dimer inhibits *in vitro* the Ran-GAP activity of Rna1 via Yrb2, a nuclear regulator of Ran GTPase (Wang et al., 2005). Gtr1-GTP has also been found to interact with Rpc19, a common subunit of RNA polymerase I, II and III. Taken together with the observation that either loss of Gtr1 or expression of a Gtr1 nucleotide-free allele caused reduced tRNA and ribosomal RNA synthesis, the authors propose that Gtr1 is involved in assembly of RNA polymerase I, II and III (Todaka et al., 2005). However, low levels of tRNA and ribosomal RNA synthesis can be an indirect consequence, since both processes are regulated by TORC1 and the absence of Gtr1 or expression of the inactive form of Gtr1 cause low TORC1 activity. Finally, it has been proposed that Gtr1 and Gtr2 interact with chromatin remodeling

factors such as Rub1, Rub2, and Ino80 to epigenetically control the TORC1 transcriptional program (Sekiguchi et al., 2008). In this study, microarray data show that Gtr1 and Gtr2 are required to repress Nitrogen Catabolite Repression (NCR) genes. Again, this effect could be rather indirect, as TORC1 is known to repress NCR and TORC1 activity depends on Gtrs.

b) The EGO/Ragulator complex

Rag GTPases form the core switch to activate TORC1 in response to amino acids. However they act as part of a larger protein complex. In *S. cerevisiae*, Gtr1 and Gtr2 associate with Ego1 and Ego3 to form the Exit from rapamycin-induced GrOwth arrest (EGO) complex (Binda et al., 2009; Dubouloz et al., 2005; Gao and Kaiser, 2006). Indeed, mutants lacking any of the EGOC subunits are unable to recover growth after rapamycin treatment (Binda et al., 2009; Dubouloz et al., 2005). This likely is due in part to the fact that these mutants cannot induce microautophagy. Hence, after rapamycin treatment, they are not able to counterbalance the massive membrane influx toward the vacuole caused by macroautophagy (Dubouloz et al., 2005). In line with this phenotype, EGOC mutants have low TORC1 activity (Binda et al., 2009). It has initially been proposed that the EGOC indirectly regulates TORC1 via the sorting of the General Amino-acid Permease Gap1 (Gao and Kaiser, 2006). However, the EGOC is required for TORC1 activity control even under conditions where Gap1 is repressed and not sorted to the plasma membrane (*i.e.* good nitrogen conditions) (Binda et al., 2009). Furthermore, cells lacking Gap1 have normal TORC1 activity and normally recover after rapamycin treatment (Binda et al., 2009). The currently accepted consensus in the field is that the EGOC directly binds and stimulates TORC1 (Fig.4) (Binda et al., 2009; Sancak et al., 2010).

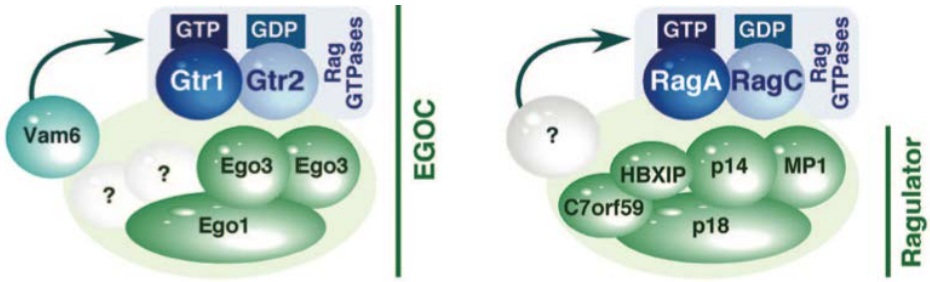

Fig. 7 Evolutionary conservation of the EGO complex. *S. cerevisiae* EGO complex is represented on the left and human Ragulator on the right. Orthologous proteins occupy corresponding positions in both schemes. Adapted from (Panchaud et al., 2013).

Ego1 is N-terminally myristoylated and palmitoylated (Ashrafi et al., 1998; Nadolski and Linder, 2009), and thereby anchors the entire EGOC to the vacuolar membrane (Binda et al., 2009; Dubouloz et al., 2005; Gao et al., 2005). Ego3 forms a homodimer that is required for the docking of Gtr1 and Gtr2 to Ego1 (Fig.7) (Zhang et al., 2012). Interestingly, the Ego3 dimer harbors a roadblock conformation similar to structure formed by the C-terminal domains of the Gtrs (Fig.8) (Zhang et al., 2012). Despite weak similarity in their primary sequence, MP1 and p14 form a heterodimer (Lunin et al., 2004) that is the human structural counterpart of the Ego3 dimer (Fig.8) (Kogan et al., 2010; Zhang et al., 2012). The MP1-p14 dimer is anchored to the lysosomal membrane via p18. Similarly to Ego1, N-terminal myristoylation and palmitoylation tether p18 to the lysosomal surface (Nada et al., 2009; Wunderlich et al., 2001). Together MP1, p14 and p18 are essential to recruit Rag GTPases to the lysosomal surface (Fig.7) and to properly relay amino acid signaling to mTORC1. Hence, the complex was named Ragulator (Sancak et al., 2010). Ragulator does not exclusively serve as scaffold for TOR signaling since it also anchors MEK-ERK pathway components to the surface of the late endosomes/lysosomes (Lunin et al., 2004; Nada et al., 2009; Schaeffer et al., 1998; Teis et al., 2006; 2002; Wunderlich et al., 2001).

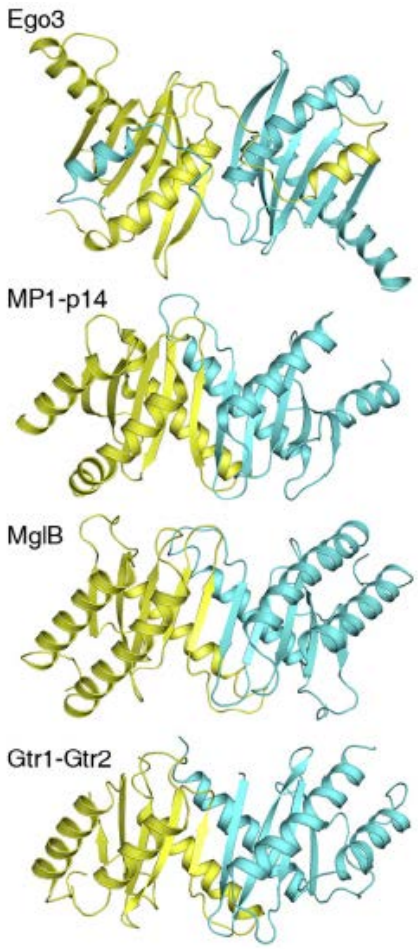

Fig. 8 Structural comparison of Roadblock domain-containing proteins. Ribbon representation of the Ego3 dimer, MP1-p14 heterodimer, the bacterial GAP MglB homodimer and Gtr1-Gtr2 C-terminal domains. Taken from (Zhang et al., 2012).

c) Regulation of Rag GTPases

The discovery that the Rag GTPases are involved in amino acid sensing raised many exciting questions : How does the EGOC/Ragulator complex sense amino acids? Which pools of amino acids are sensed among extracellular, cytoplasmic and vacuolar/lysosomal amino acids? What are the primary sensors of amino acids? What are the GEFs, GAPs and GDIs regulating Rags? Over the last few years, efforts by several groups have provided parts of the answers and revealed that a complex signaling network takes place upstream of the Rag GTPases.

1) Ragulator is a GEF for RagA/B

An expanded version of Ragulator, which in addition to MP1, p14 and p18 contains HBXIP and C7orf59, exhibits GEF activity toward RagA and B but not RagC (Fig.9)

(Bar-Peled et al., 2012). Like MP1 and p14, HBXIP and C7orf59 also contain Roadblock domains. A previous study reported that Ragulator constitutively interacts with Rag GTPases (Sancak et al., 2010). However Bar-Peled *et al.* show that expanded Ragulator interacts with Rags in an amino acid dependent manner and activates RagA/B in the presence of amino acids. The entire Ragulator is required for GEF activity *i.e.* neither HBXIP-C7orf59 nor MP1-p14-p18 alone display GEF activity. Surprisingly, the pentameric Ragulator does not contain any domain resembling a known GEF catalytic domain. Whether the EGO complex assumes the same role in yeast is not known. Although no clear orthologs of HBXIP and C7orf59 have been identified in *S. cerevisiae*, a bioinformatic analysis predicted that two uncharacterized proteins, Ycr075w and Ynr034w, may be structural homologs of HBXIP and C7orf59 (Levine et al., 2013). However no evidence exists to link these proteins to TORC1 signaling so far.

2) Vam6 is a GEF for Gtr1

In *S. cerevisiae*, Vam6 exerts a GEF activity toward Gtr1 (Binda et al., 2009). Vam6 is part of the HOmotypic fusion and vacuole Protein Sorting (HOPS/Class C-vps) complex, a tethering complex involved in vesicle fusion (Caplan et al., 2001; Ostrowicz et al., 2008). CORVET and HOPS Class C-vps complexes play an important role in endolysosomal trafficking and are required for proper TORC1 signaling (Zurita-Martinez et al., 2007). Class C-vps mutants cannot maintain amino acid homeostasis, which results in TORC1 inactivation. This can be partially rescued by either the addition of extracellular amino acids or by expression of the active forms of the Gtr proteins (Kingsbury et al., 2014). In *S. pombe* Vam6 also interacts with Gtr1 and is required for its activation (Valbuena et al., 2012). In human cells knockdown of the Vam6 ortholog hVps39 results in low mTORC1 activity (Flinn et al., 2010). However mTORC1 localization is not impaired, indicating that the RagA/B nucleotide-binding status may not be influenced by hVps39. Moreover, hVps39 did not exert any GEF activity on RagB *in vitro* (Bar-Peled et al., 2012). Humans have another Vam6 ortholog coined TGFβ Receptor-Associated Protein 1 (TGFβRAP1 or TRAP1) which is essential for early embryonic development (Messler et al., 2011). It would therefore be interesting to assess the involvement of this protein in mTORC1 signaling and to test its potential GEF activity on RagA/B (Fig.9).

3) The v-ATPase

The amino acid sensitive branch of TORC1 signaling localizes at the vacuolar/lysosomal periphery. Strikingly, these organelles are the main amino acid reservoirs in the cell (Wiemken Arch Microbiol 1974). Acidification of the vacuole is essential for uptake of amino acids inside the vacuole. The low vacuolar pH (between 4.6 and 5) is maintained by the highly conserved vacuolar H^+-ATPase (v-ATPase), a proton-pump that hydrolysis ATP to import protons inside the vacuolar lumen (Mellman et al., 1986). The v-ATPase is a multiprotein complex composed of two domains: i) the V0 domain, which is composed of eight subunits (A to H) and responsible for the translocation of protons from the cytoplasm into the vacuolar lumen. ii) The V1 domain containing six subunits (a, d, c, c', c'', and e), that hydrolyses ATP to fuel proton transport (Forgac, 2007). In flies and mammals, the v-ATPase V1 domain interacts with Rag GTPases and both V0 and V1 domains interact with Ragulator (Zoncu et al., 2011). This interaction is dependent on amino acids: deprivation of amino acids strengthens the V1 interaction with Ragulator. The genetic or chemical disruption of v-ATPase function strongly reduces the ability of Rag GTPases to activate mTORC1 in response to amino acids. V-ATPase does not regulate the localization of the Rag GTPses, but rather acts on Ragulator GEF activity (Zoncu et al., 2011). It is not clear how the v-ATPase senses amino acids but it has been proposed that the lysosomal pool of amino acids, rather than the proton gradient, is particularly important for this process. Addition of amino acids to a cell-free system containing purified lysosomes, Rags and mTORC1 components, was able to promote the Rags-Raptor interaction, indicating that the lysosome contains all the machinery required for mTORC1 activation in response to the addition of amino acids (Zoncu et al., 2011). Furthermore, decreasing the lysosomal amino acid level by overexpression of the amino acid transporter PAT1 (but not disruption of the proton gradient with an ionophore) suppressed mTORC1 activation by amino acids (Zoncu et al., 2011). Thus the authors proposed an inside-out mechanism where the v-ATPase senses intra-lysosomal amino acids and modulates mTORC1 activity outside of lysosome via the Ragulator-Rags complex (Fig.9). This model does neither take into account the cytosolic pH nor the levels of cytosolic amino acids. However, two reports show that reduced cytosolic pH due to v-ATPase impairment causes a reduction in mTORC1 activity (Balgi et al., 2011; Fonseca et al., 2012). Whether the v-ATPase also regulates TORC1 in yeast remains to be determined but, interestingly,

Ego1 is required for proper vacuolar acidification and stabilization of the v-ATPase component Vma2 (Gao et al., 2005).

4) Leucyl-tRNA Synthetase

A cytosolic sensor of intracellular leucine is the Leucyl-tRNA Synthetase (LeuRS), an enzyme that mediates ligation of L-leucine to its corresponding tRNA (Ling et al., 2009). In yeast, LeuRS binds and regulates Gtr1. In the presence of leucine, the LeuRS editing domain interacts with Gtr1 and promotes its GTP-bound form, possibly by blocking hydrolysis. Under leucine deprivation, LeuRS is more likely to mischarge $tRNA^{Leu}$ with the incorrect amino acids and requires the engagement of its editing domain to correct the mistake. This causes a conformational change of the domain that may no longer able to protect Gtr1-GTP from hydrolysis (Bonfils et al., 2012). Human Leucyl-tRNA Synthetase also communicates leucine availability to TORC1 through the Rag GTPases (Fig.9). However it has been proposed that LeuRS acts as a GAP for RagD, promoting its GDP-bound, active form. A domain in the leucine-binding pocket would trigger the GAP activity through the arginine 845 (Han et al., 2012). It cannot be excluded that LeuRS exerts a dual activity on Rag GTPases: on one hand it protects RagA/B-GTP from hydrolysis via its editing domain, and on the other it promotes RagC/D hydrolysis via its leucine-binding pocket GAP domain. Another possibility is that the mechanism by which LeuRS regulates Rags diverged through evolution. The fact that the catalytic arginine is not conserved in *Drosophila* or in yeast, despite the high degree of similarity of LeuRS across different species, supports the latter notion. Intriguingly, the LeuRS GAP domain harbors a motif surrounding the catalytic arginine that is similar to ArfGAPs (Han et al., 2012). The structure of Gtr2 revealed that its G-domain is different from those found in the Arf GTPases and that a Gtr2/RagC/D GAP may not necessarily need to provide an arginine finger as the Gtr2 P-loop contains an arginine able to stabilize the transition state (Jeong et al., 2012). Finally, another group failed to show GAP activity of LeuRS on RagC and D (Tsun et al., 2013).

5) Folliculin

Recently, the Folliculin (FLCN) tumor suppressor has been identified as a regulator of Rag proteins. Together with its binding partner FNIP1, FLCN is required for proper

TORC1 relocalisation to the lysosomal surface and its activation in presence of amino acids (Petit et al., 2013; Tsun et al., 2013). FNIP1 recruits FLCN to the lysosomal membrane after amino acid deprivation, where the complex interacts with the Rag GTPases. As is the case for Ragulator and the v-ATPase, the interaction between the Rag heterodimer and folliculin is strengthened upon amino acid starvation (Petit et al., 2013; Tsun et al., 2013). FLCN directly binds the G domain of RagA but not RagC. This observation led Petit *et al.* to propose that folliculin acts as a GEF for RagA/B. Such a hypothesis is tempting, since FLCN and FNIP1 contain DENN domains that are typically found in GEFs (Levine et al., 2013). Tsun *et al.* also noticed that FLCN preferentially binds to the nucleotide-free form of RagB, but did not detect any GEF activity of the FLCN-FNIP1 complex toward the Rag GTPase. It rather seems that the FLCN-FNIP1 complex use RagA/B as docking site to then exert GAP activity on RagC/D (Fig.9) (Tsun et al., 2013). Thus FLCN-FNIP1 promotes the active GDP-bound form of RagC/D and subsequent recruitment of mTORC1 at the lysosome for activation. These new findings raise several questions: i) Why does FLCN-FNIP1 relocalize to the lysosomal surface and interact with Rags upon amino acid starvation? As a positive regulator we would expect that the complex would colocalize with Rags in presence of amino acids. ii) Why does a tumor suppressor positively regulate mTORC1? One possible explanation could be that suppression of mTORC1 activity leads to over-activation of other pathways, such as the PI3K/Akt signaling pathway. Whether a similar mechanism exists in yeast remains to be determined. Of note, FLCN and FNIP1 orthologs Lst7 and Lst4 share the same sensitivity to various chemical and environmental insults as the EGO complex components (Hillenmeyer et al., 2008).

6) MAP4K3

In humans and flies, the kinase MAP4K3 is a positive regulator of mTORC1 in response to amino acid availability (Findlay et al., 2007; Yan et al., 2010). Amino acids induce phosphorylation of serine 170 in MAP4K3 (Yan et al., 2010). As a consequence, activated MAP4K3 stimulates mTORC1 in a Rag dependent manner (Bryk et al., 2010; Yan et al., 2010). MAP4K3 physically interacts with Rags and preferentially bind to RagC-GDP (Fig.9) (Bryk et al., 2010). Upon amino acid starvation PP2A dephosphorylates MAP4K3 ser170 (Yan et al., 2010) and the interaction between Rags and MAP4K3 is decreased (Bryk et al., 2010). An

interesting model would be that MAP4K3 modulates Rags activity through direct phosphorylation. However mutation of a RagC known phosphosite (S388E) did not alter its binding with MAP4K3 (Bryk et al., 2010). It also remains to be determined how the Rags-MAP4K3 interaction is regulated by amino acids. Mutation of MAP4K3 serine 170 to non-phosphorylable (alanine) or phosphomimetic (glutamic acid) residues did not alter the Rags-MAP4K3 interaction. Hence, the S170 phosphorylation state could regulate MAP4K3 activity rather than substrate interaction. Resnik-Docampo *et al.* confirmed that MAP4K3 physically interacts with Rags in *Drosophila* but proposed that MAP4K3 acts downstream of Rags to regulate TORC1 assembly (Resnik-Docampo and de Celis, 2011). It would be interesting to assess whether the yeast counterpart of MAP4K3 could be the kinase Ste20, which is involved in mating pheromone response pathway, or the putative kinase Sps1.

7) Glutaminolysis

Glutamine and glutamate plays a very important role in cells as precursors of α-ketoglutarate, an essential component for the tricarboxylic acid (TCA) cycle. Glutaminase converts glutamine to glutamate, and glutamate can be further converted to α-ketoglutarate by the glutamate dehydrogenase (Curi et al., 2005). This process is called glutaminolysis and can be sensed by mTORC1 through the Rag GTPases (Durán et al., 2012). Interestingly, the potent TORC1 activator leucine binds to glutamate deshydrogenase and stimulates glutamate deamination and thereby induces production of α-ketoglutarate (Sener and Malaisse, 1980). How glutaminolysis regulates Rags is not clear but it may involve a prolyl hydroxylase (PHD) in a mechanism that ultimately promotes GTP loading of RagB (Fig.9) (Durán et al., 2012). Such a mechanism has not been investigated in yeast, but glutamine is known to be a potent TORC1 activator (Dubouloz et al., 2005).

8) Glutamine-Leucine antiporter

In humans another mechanism involving glutamine activates mTORC1. Non-essential intracellular glutamine is exported through the SLC7A5/SLC3A2 antiporter complex, allowing influx of essential leucine inside the cell (Nicklin et al., 2009). Glutamine used in this process can be provided either by *de novo* synthesis or by import through the SLC1A5 transporter. Individual knock-down of SLC7A5, SLC3A2

or SLC1A5 is sufficient to impair leucine import and subsequent mTORC1 activation (Fig.9) (Nicklin et al., 2009).

9) Proton-assisted amino-acid transporters

In flies and humans, two Proton-assisted Amino-acid Transporters, PAT1 and PAT4 are implicated in Rags activation. PAT1 and PAT4 shuttle between the plasma membrane and late endosome/lysosome. PAT1 mainly localizes to the lysosomal membrane, where it is able to interact with Rags and to provoke mTORC1 recruitment upon amino acid stimulation (Fig.9) (Heublein et al., 2010; Ögmundsdóttir et al., 2012). The amino acid transport function of PAT1 should be active at the late endosome/lysosome rather than at the plasma membrane, as a proton gradient is required for co-transport (Heublein et al., 2010). Whether PATs activate Rags directly or indirectly through augmentation of the cytoplasmic amino acids level is not clear. PATs are involved in the transport of small amino acids such as alanine, proline and glycine, which are not known to be strong TORC1 activators. Interestingly, stimulation of the PI3K/Akt/Rheb signaling branch of mTORC1 promotes PATs endocytosis (Ögmundsdóttir et al., 2012). We can therefore imagine a mechanism where in the presence of growth factors, but low cytosolic amino acid levels, PATs would export amino acids stored in the lysosome to fully activate mTORC1. Rab GTPases are central regulators of the membrane transport pathway. Rab12 has been identified as a negative regulator of PAT4 (Matsui and Fukuda, 2013). Rab12 stimulates PAT4 degradation thereby decreasing the import of amino acids into the cytosol and consequently downregulating mTORC1 activity. In their model, the authors propose that PAT4 is active at the plasma membrane rather than at the lysosome (Matsui and Fukuda, 2013). Like PAT1, PAT4 should need a proton gradient to co-transport amino acids, however a recent report indicates that when expressed in *Xenopus laevis* oocytes PAT4 is more active at neutral pH than at low pH (Pillai and Meredith, 2011). Furthermore Matsui *et al.* mainly localized PAT4 at the plasma membrane. Therefore it is possible that PAT4 is mostly active at the plasma membrane whereas PAT1 is in charge of lysosomal to cytoplasm amino acid transport. This would explain the lack of redundancy on mTORC1 inhibition observed by Heublein *et al.* in PAT1 or PAT4 knockdown cells. More work is needed to understand the link between PATs, vATPase, lysosomal amino acids and mTORC1. A simple model would be that PAT1 exports amino acids from the lysosome while the

vATPase pumps protons inside the lysosome to maintain the pH gradient necessary for PAT1 co-transport. When the system is active PAT1 or the vATPase or both adopt a conformation that interacts with and activates RAGs (Ögmundsdóttir et al., 2012).

10) T1R1/T1R3 G protein-coupled receptor

In humans, the G protein-coupled receptor T1R1/T1R3 has been implicated in mTORC1 activation in response to amino acid stimulation (Wauson et al., 2012). This model is interesting as T1R1/T1R3 can directly bind amino acids and act as extracellular sensor. However it is not clear if Rag GTPases have a direct involvement in relaying the signal. On one hand, cells in which T1R1/T1R3 are disrupted are unable to properly relocalize mTORC1 to the lysosome in the presence of amino acids, indicating that Rags are likely to be misregulated. On the other hand, T1R1/T1R3 signals through ERK-MEK, a module that is known to impinge on the TSC complex (Fig.9) (Ma et al., 2005). Of course one mechanism does not exclude the other, but further studies are needed to decipher how the T1R1/T1R3 receptor communicates extracellular amino acid status to mTORC1. An interesting model would be that T1R1/T1R3 impinges on the Ragulator complex. Ragulator is required for proper localization and function of Rag GTPase and MEK-ERK signaling components (Lunin et al., 2004; Nada et al., 2009; Sancak et al., 2010; Schaeffer et al., 1998; Teis et al., 2002; 2006; Wunderlich et al., 2001). Hence we can imagine that in presence of external amino acids T1R1/T1R3 would promote Ragulator interaction with Rags and MEK-ERK components allowing full activation of both pathways.

11) Rags modulators

Different modulators of the interaction between Rags and mTORC1 have been identified in human cells. The adaptor protein p62 is required for proper amino acid signaling to mTORC1. p62 interacts with mTORC1 and the Rag active heterodimer via RagC (Fig.9). It can act as a scaffold to stabilize the active Rag dimer (Duran et al., 2011). The Inositol Polyphosphate Multikinase (IPMK) modulates the strength of interactions between mTORC1 components. In the presence of amino acids, IPMK weakens the interactions inside mTORC1, thereby facilitating its interaction with

Rags and Rheb for activation (Fig.9) (Kim et al., 2011). Upon amino acid starvation, SH3BP4 directly binds and stabilizes the Rag inactive heterodimer through its SH3 domain. SH3BP4 binding to Rags abrogates the Rag-mTORC1 interaction and inhibits mTORC1 recruitment to the lysosomal membrane. It also blocks the formation of an active Rag GTPase complex as it prevents GTP binding to RagB (Fig.9) (Kim et al., 2012). More work is needed to understand how these modulators are regulated and how they mechanistically influence Rags activity and binding with mTORC1. So far Rags regulation by p62, IPMK and SH3BP4 seems to be restricted to mammals.

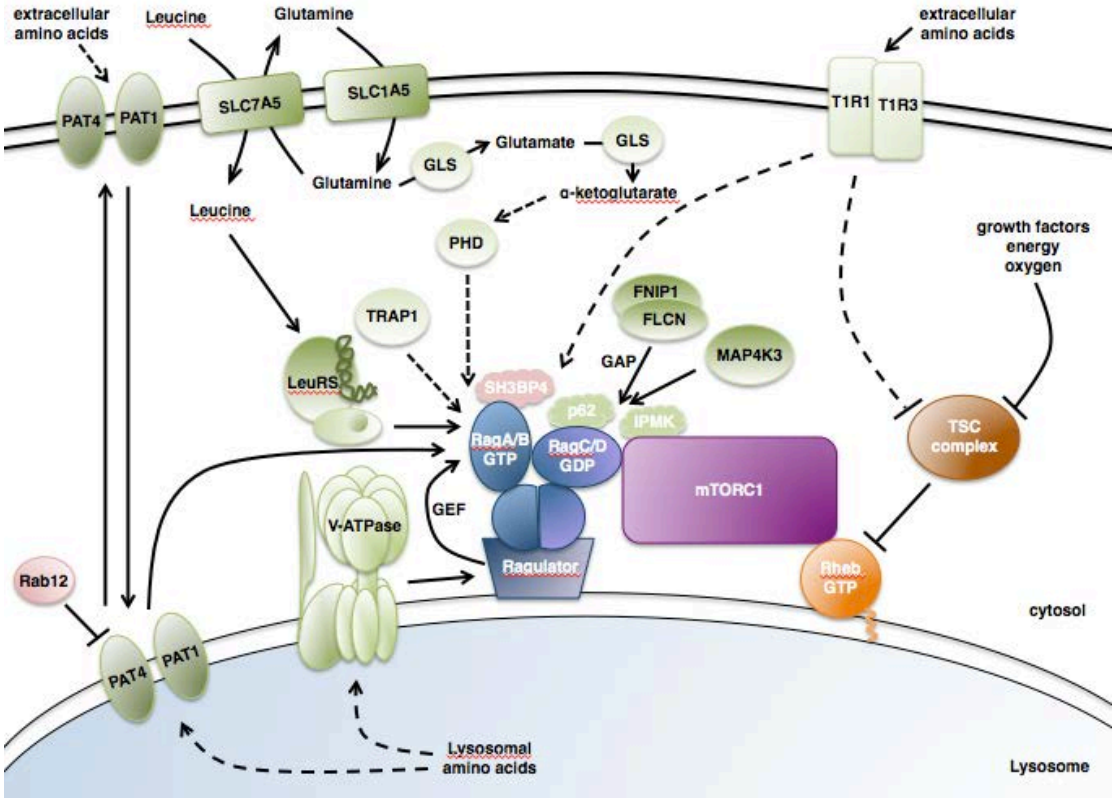

Fig. 9 Regulation of Rag GTPases. The Rag-Ragulator complex is represented in blue. Positive regulators of Rag GTPases are in green and negative regulators in red. The Rheb-TSC module is represented in orange and the TOR complex in purple. Arrows show activation events and bars indicate inhibitions. Dashed lines represent indirect and/or potential interactions, whereas solid lines denote direct interactions. See text for more details.

Aim and Outline

The aim of this thesis is to understand how amino acids regulate the EGOC and in particular how they modulate the nucleotide loading status of the Gtr's heterodimer. Most of the Rag GTPase regulators identified so far are positive components of the pathway. Negative regulators, like a GAP for RagA/B/Gtr1, remained elusive. In the first chapter of this thesis, I will focus on the recently identified SEA complex (SEAC). We found that SEAC is composed of two subcomplexes with distinct functions: SEACIT, a trimeric protein complex that negatively regulates TORC1 in response to amino acids starvation, and SEACAT, a pentameric complex that negatively regulates SEACIT. One of the SEACIT components, Iml1, acts as a GAP on Gtr1.

In the second chapter I will investigate the discrepancy between yeast and mammals in the activation mode of RagA/B/Gtr1. Yeast Vam6 act as a GEF on Gtr1, but in mammals an extended version of Ragulator is the GEF. Here we identified a new component of the EGOC, which is likely a functional ortholog of HBXIP and C7orf59. We named this component Ego2 and demonstrate that it is essential for EGOC integrity. However, we were not able do show any *in vitro* GEF activity of the extended Ego1-Ego2-Ego3 complex on Gtr1 so far.

Finally, in the third chapter I will discuss the effects of different small molecules on TORC1 activity and their therapeutic potential.

Chapter I :

Amino Acid Deprivation Inhibits TORC1 Through SEACIT, a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr1

Part of the work from this chapter has been published in
Science Signaling, Vol. 6 (issue 277) and Cell Cycle, Vol. 12 (issue 18).

1.1 Introduction

Several activators of Rag GTPases have been identified so far (*i.e.* the leucyl-tRNA synthetase, the v-ATPase, the RagA/B GEF Ragulator, the Gtr1 GEF Vam6, and the RagC/D GAP FLCN-FNIP1/2 complex). However, negative regulators such as a putative GEF for RagC/D or Gtr2, or a GAP that suppresses the activity of RagA, RagB, or Gtr1 have yet to be identified. A genome-wide screen for regulators that inhibit TORC1 in response to amino acid deprivation identified the yeast proteins Npr2 and Npr3 (Neklesa and Davis, 2009), which have later been found to be part of a large vacuolar membrane-associated complex together with Iml1, Sea2, Sea3, Sea4, Seh1 and Sec13, coined SEA complex (SEAC) for *Se*h1-*a*ssociated complex (Dokudovskaya et al., 2011). Structure predictions show that SEAC shares features with membrane trafficking complexes such as COPI/II and the class C vps complexes HOPS and CORVET (Dokudovskaya et al., 2011). The latter study also linked Npr2 and Npr3 to autophagy. Another group proposed that Iml1, Npr2, and Npr3 form a complex that promotes autophagy, although independently of TORC1 (Wu and Tu, 2011). Another discrepancy between the two studies concerned the localization of SEAC: Wu and Tu observed the Iml1-Npr2-Npr3 complex in punctate structures that they identified as the pre-autophagosome structure (PAS), whereas (Dokudovskaya et al., 2011), reported that SEAC localized at the vacuolar rim. In this chapter the role of the SEAC and the nature of its relations with TORC1 will be deciphered.

1.2 Results

a) SEAC is composed of two sub-complexes regulating TORC1 in an opposite manner

To clarify the role of SEAC in TORC1 regulation, we assessed TORC1 activity in cells lacking individual components of the SEAC, all of which grew normally at 30°C and responded properly to leucine starvation in terms of phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), a sensitive indicator of the presence of uncharged tRNAs that stimulate the eIF2α-kinase Gcn2 (Hinnebusch, 2005) (Fig. 1.1A and B). Loss of Iml1, Npr2, or Npr3, but not of Sea2, Sea3, or Sea4 resulted in increased TORC1 activity, as assessed by phosphorylation of Sch9 (Fig. 1.1C and D). Together with the observation that the concomitant loss of either Npr2 and Npr3, or of Npr2, Npr3, and Iml1 stimulated TORC1 activity similarly (Fig. 1.2A), these data indicate that Npr2, Npr3, and Iml1 share a common biological function in inhibiting TORC1.

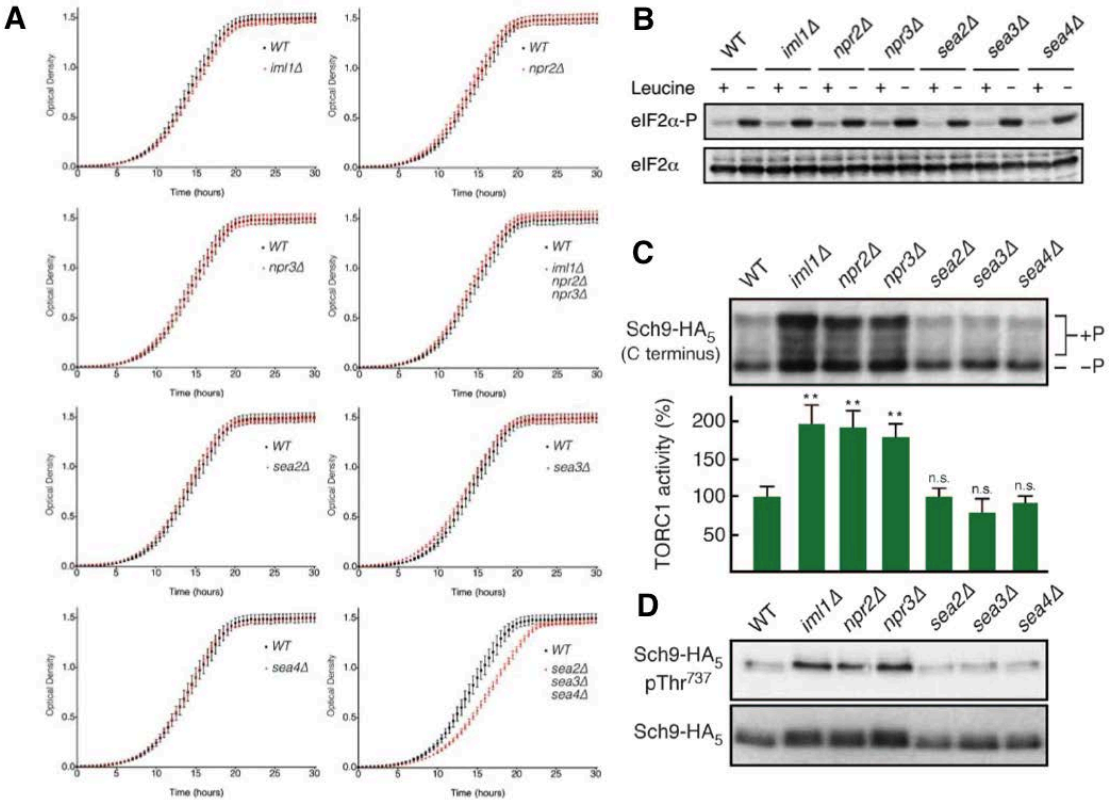

Fig.1.1 The vacuolar membrane-associated Iml1·Npr2·Npr3 complex inhibits TORC1. (A, B) Loss of individual SEA complex subunits does not substantially affect growth of cells at 30°C or eIF2a phosphorylation following leucine starvation. (A) Optical density was measured at 600 nm. As expected based on their intrinsically low TORC1 activity (Fig. 1.2A), triple *sea2Δ sea3Δ sea4Δ* mutant cells exhibited a slow growth phenotype (lower panel on the right). Data are means ± S.D. from three independent experiments. (B) Phosphorylation of eIF2a (on Ser[51]; eIF2a-P) was assessed as previously

described (Bonfils et al., 2012) prior to (+) and following (-) a 60-min period of leucine starvation. One representative immunoblot from three independent experiments is shown. **(C, D)** Loss of Iml1, Npr2, or Npr3, but not of Sea2, Sea3, or Sea4 causes an increase in TORC1 activity. (C) Immunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 were used to quantify TORC1 activity (the ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9) (Urban et al., 2007) . The values were normalized to the ones for wild-type (WT) cells and presented in the bar graph as means (+ S.D.; n=3 independent experiments). (D) Similar results for TORC1 activities (ratio of $pThr^{737}$/total full length Sch9) were obtained by using specific antibodies recognizing the phosphorylated Thr^{737} ($pThr^{737}$) of Sch9 (Takahara and Maeda, 2012), a major TORC1 target in yeast (Urban et al., 2007). One representative immunoblot from three independent experiments is shown. $^{**}P < 0.01$, n.s., not significant in Student's *t*-test compared to respective wild-type control (*P*-values are Holm-Bonferroni adjusted).

In contrast, Seh1, Sec13, or Sea2-Sea3-Sea4 in combination, have a stimulatory activity toward TORC1. Loss of *SEH1*, or expression of a *SEC13* thermo-sensitive allele at permissive temperature (25°C), or deletion of *SEA2*, *SEA3*, and *SEA4* combined, decreased TORC1 activity in an Iml1-, Npr2-, and Npr3-dependent manner (Fig. 1.2A, B and C). This indicates that the SEAC is subdivided in two functionally different subcomplexes: the Seh1-Sec13-Sea2-Sea3-Sea4 subcomplex that acts upstream of and inhibits the Iml1-Npr2-Npr3 subcomplex, which is itself an inhibitor of TORC1. We therefore named the Iml1-Npr2-Npr3 subcomplex SEACIT (for SEAC subcomplex Inhibiting TORC1 signaling) and the Seh1-Sec13-Sea2-Sea3-Sea4 subcomplex SEACAT (for SEAC subcomplex Activating TORC1 signaling).

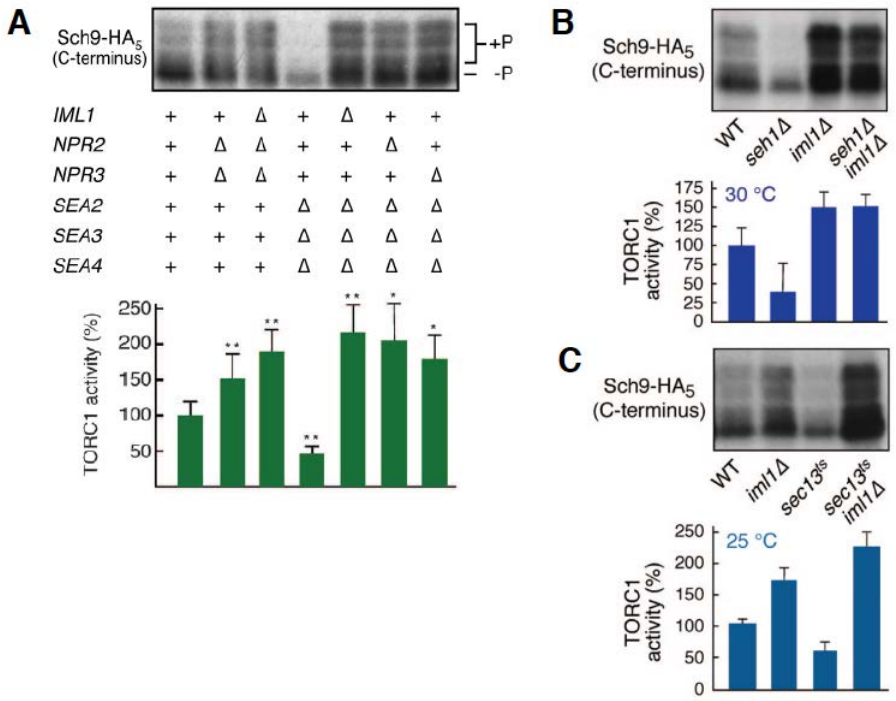

Fig. 1.2 Two subcomplexes with an antagonistic effect on TORC1 form the SEAC. (**A, B, C**) Effects on TORC1 activity of various combinations of individual deletions (Δ) of SEA complex subunit-encoding genes. One Immunoblot out of 3 is shown; quantifications were done as described in 1.1C. $^{**}P < 0.01$, $^{*}P < 0.05$, n.s., not significant in Student's *t*-test compared to respective wild-type control (*P*-values are Holm-Bonferroni adjusted).

To further dissect the roles of the individual components of SEACIT, we overproduced Iml1, Npr2, Npr3, or Npr2 together with Npr3, and examined the corresponding effects on TORC1 activity in wild-type, *iml1Δ*, *npr2Δ,* and *npr3Δ* strains. Overproduction of Iml1 bypassed the requirement for Npr2 or Npr3 to decrease the activity of TORC1, but not *vice versa* (Fig. 1.3A). Consistent with these genetic data, Iml1 did not require Npr2/3 or Sea2/3/4 to be recruited to the vacuolar membrane (Fig. 1.3B and C), whereas Npr2 and Npr3 depended on each other and on Iml1 for their localization to the vacuolar membrane. This led us to speculate that the functional roles within SEACIT may be partitioned into a catalytic role for Iml1 and structural and/or regulatory roles for Npr2 or Npr3.

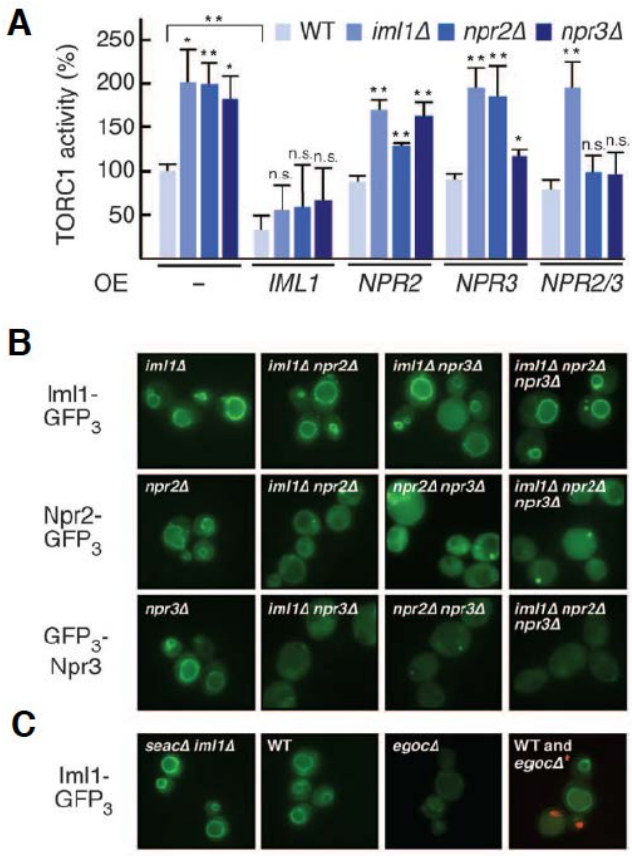

Fig. 1.3 Iml1 plays a central role in the Iml1-Npr2-Npr3 complex. (**A**) Effects on TORC1 activity of overexpression (OE) of *IML1* or of *NPR2* and *NPR3* (individually or in combination) in different mutant backgrounds. Data are means + S.D. from three independent experiments. **(B,C)** Wild-type or mutant strains expressing the indicated functional GFP-fusion proteins from their own promoter were analyzed by fluorescence microscopy during exponential growth. In (C) *egocΔ* and *seacΔ* denote the *gtr1/2Δ ego1Δ ego3Δ* and *sea2-4Δ* mutants, respectively. Wild-type and *egocΔ** (expressing the nuclear marker Hhf2-RFP) cells were mixed to directly compare Iml1-GFP_3 signals at the vacuolar membrane. Representative images are shown from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, n.s., not significant in Student's *t*-test compared to respective wild-type control (*P*-values are Holm-Bonferroni adjusted).

b) SEACIT negatively regulates TORC1 through the EGO complex

Our cell biological analyses revealed that Iml1 required the presence of the EGO complex (comprising Gtr1 and Gtr2 and their vacuolar membrane anchors Ego1 and Ego3; (Dubouloz et al., 2005) to be efficiently localized at the vacuolar membrane (Fig. 1.3C). This suggested that Iml1 may regulate TORC1 through the Gtr1-Gtr2 heterodimer. Consistent with this idea, the absence of Gtr1 or Gtr2 (or of the TORC1 subunit Tco89; Fig. 1.4A), or the expression of signaling-compromised $Gtr1^{S20L}$ (which has low affinity for nucleotides) or GTP-locked $Gtr2^{Q66L}$ (Fig. 1.4B), prevented the increase in activation of TORC1 in *iml1Δ* cells. Elevated TORC1 activation in *npr2Δ* and *npr3Δ* cells was also dependent on Gtr1 (Fig. 1.4A). Moreover, expression of the GTP-locked, signaling-competent $Gtr1^{Q65L}$ allele and loss of Iml1 both individually and in combination stimulated TORC1 to a similar extent, indicating that Iml1 may in fact specifically function upstream of and inhibit Gtr1 (Fig. 1.4B). Two additional observations support this notion: (i) TORC1 activity remained sensitive to loss or overproduction of Iml1 in the presence of $Gtr2^{S23L}$, which has low affinity for nucleotides (Fig. 1.4B and C), but (ii) was largely refractory to Iml1 overproduction in $Gtr1^{Q65L}$ or $Gtr1^{S20L}$ expressing cells (Fig. 1.4C). Lastly, as predicted by a model in which SEACIT decreases the activity of Gtr1 following amino acid deprivation, loss of Iml1, or of Npr2 and Npr3 (individually or in combination), like overexpression of $Gtr1^{Q65L}$, rendered TORC1 activity partially resistant to leucine starvation (Fig. 1.4D and E).

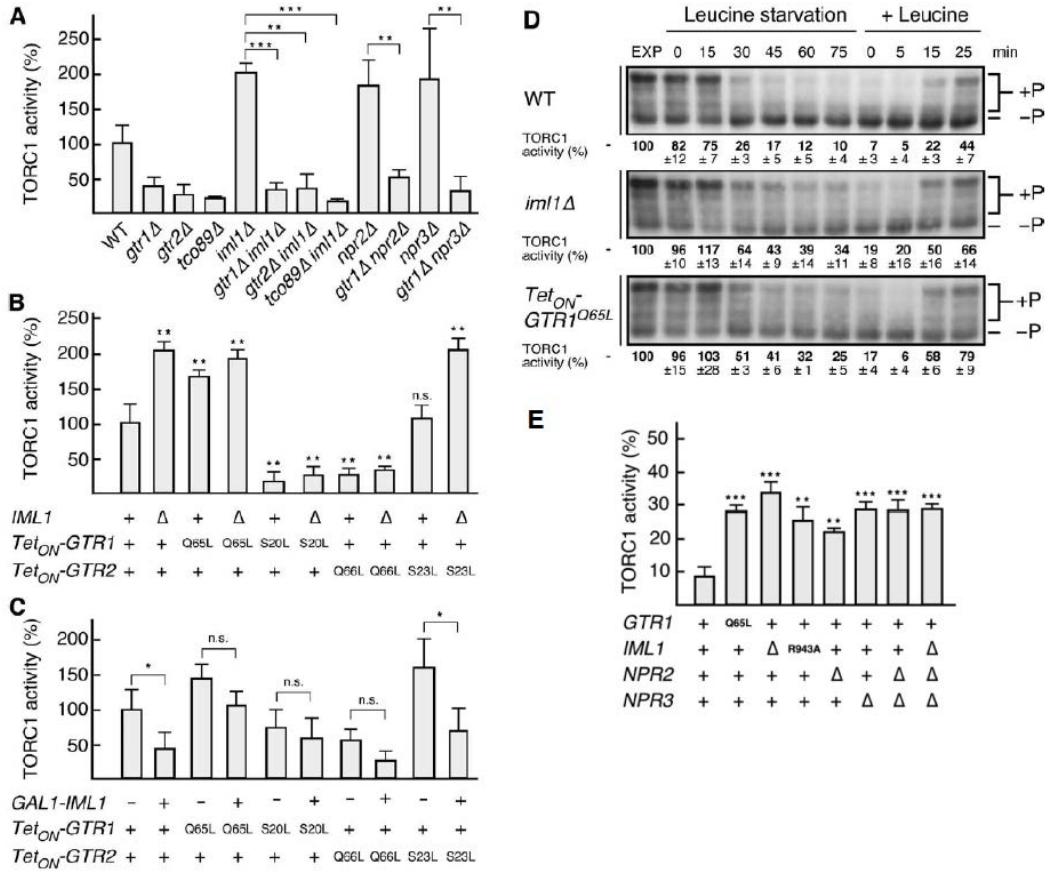

Fig. 1.4 SEACIT controls TORC1 activity through the Rag GTPase heterodimer Gtr1·Gtr2. (A) Increased TORC1 activity observed in the absence of Iml1, Npr2, or Npr3 requires the presence of Gtr1 or Gtr2 (means + S.D. from three independent experiments). . **(B, C)** Effects on TORC1 activity of loss (B) and overproduction of Iml1 (C) in the presence of overexpressed Gtr1^{Q65L}·Gtr2, Gtr1^{S20L}·Gtr2, Gtr1·Gtr2^{Q66L}, or Gtr1·Gtr2^{S23L} heterodimers. (means + S.D. from three independent experiments). **(D,E)** Loss of Iml1·Npr2·Npr3 complex subunits, individually or in combination, or expression of Gtr1^{Q65L} or of Iml1^{R943A}, renders TORC1 activity partially insensitive to leucine starvation. (D) Numbers are means ± S.D. from three independent experiments, one representative immmunoblot is shown. (E) TORC1 activities (means + S.D.; n=3 independent experiments, assayed as in Fig. 1.1C) were assessed following 30 min of leucine starvation and normalized to the respective value in each strain before leucine starvation. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s., not significant in Student's *t*-test compared to indicated control in (A) and (C), or to wild type in (B) (*P*-values are Holm-Bonferroni adjusted).

c) Iml1 interacts with Gtr1 in a leucine dependent manner.

Our genetic epistasis analyses led us to examine whether Iml1 interacted with Gtr1 in cells. Iml1 specifically bound Gtr1 in the presence, but substantially less in the absence of Npr2 and Npr3 (Fig. 1.5A and B). Moreover, although GFP-fused variants of Iml1, Npr2, and Npr3, like EGOC and TORC1 (Binda et al., 2009), all localized to the vacuolar membrane both prior to and following leucine deprivation (Fig. 1.5C), bimolecular fluorescence complementation (BiFC) analysis revealed that leucine deprivation stimulated the Iml1-Gtr1 interaction specifically at the vacuolar membrane (Fig. 1.5D). This was also readily observable in coimmunoprecipitation experiments

in which leucine deprivation transiently stimulated the Iml1-Gtr1 interaction, but not the constitutively strong Iml1-Gtr1^{Q65L} interaction (Fig. 1.5E). To explore whether Iml1 regulated the GTP-loading status of Gtr1 in cells, we made use of the fact that the TORC1 subunit Kog1 binds preferentially the GTP-bound form of Gtr1 (Binda et al., 2009); hence, the amount of Gtr1-associated Kog1 can be used as a proxy for the relative amount of Gtr1^{GTP} within cells. We observed that Iml1 overproduction reduced the Kog1-Gtr1 interaction, but not the Kog1-Gtr1^{Q65L} interaction (Fig. 1.5F), which implicates Iml1 as a potential GAP for Gtr1. In accordance with this notion, the binding of purified Iml1 to Gtr1 was enhanced by the presence of the nonhydrolysable GTP analog GTPγS or of GDP-AlF$_x$ which is a structural mimic of the transition state in the hydrolysis reaction by GTPases (Fig. 1.5G) (Wittinghofer and Vetter, 2011) and identifies interactions of GTPases with their cognate GAPs (Scheffzek et al., 1998).

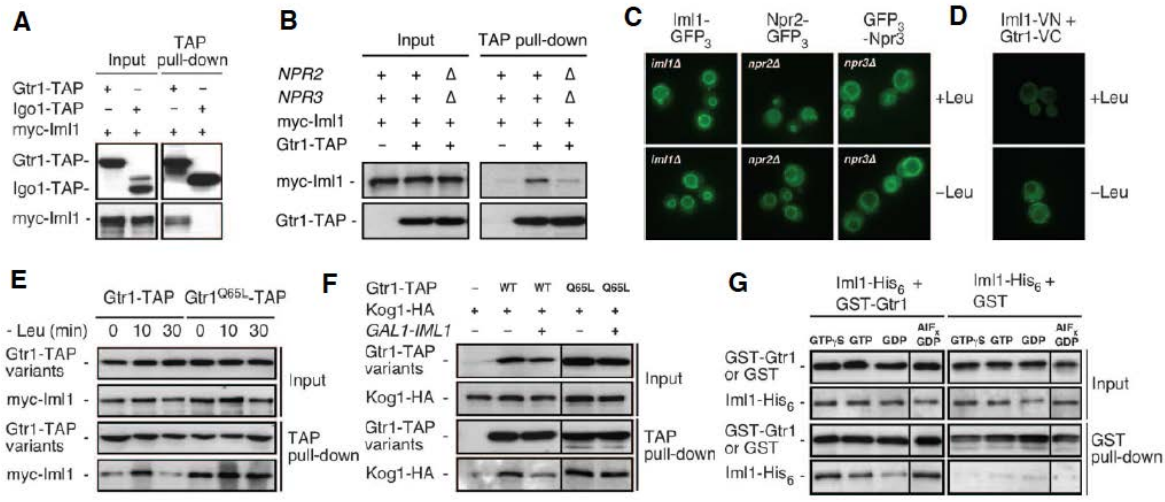

Fig. 1.5 Leucine inhibits the interaction between Iml1 and Gtr1 at the vacuolar membrane. (A, B) In exponentially growing wild-type cells myc-Iml1 physically interacts with Gtr1-TAP in the presence (A), but substantially less in the absence of Npr2 and Npr3 (B), and not at all with the control fusion protein Igo1-TAP. Lysates (Input) from exponentially growing wild-type and *npr2Δ npr3Δ* cells expressing the indicated fusion proteins and TAP pull-down fractions were analyzed by immunoblotting using anti-TAP or anti-myc antibodies. One representative immunoblot from three independent experiments is shown. **(C, D)** Leucine deprivation does not change the vacuolar membrane localization of Iml1-GFP$_3$, Npr2-GFP$_3$, and GFP$_3$-Npr3 (C), but stimulates the interaction between Gtr1 and Iml1 at the vacuolar membrane as assayed via BiFC (D), which allows detection of protein-protein interactions in cells due to reconstitution of the fluorescent Venus protein (Sung and Huh, 2007). VN and VC denote N-terminal and C-terminal fragments of Venus, respectively. Representative images are shown from three independent experiments. (**E**) TAP pulldown analyses indicate that the Iml1-Gtr1 interaction is transiently stimulated following leucine starvation, whereas Iml1 constitutively binds Gtr1^{Q65L}. **(F)** The Gtr1-Kog1 interaction, but not the Gtr1^{Q65L}-Kog1 interaction, is reduced when *IML1* is overexpressed from the *GAL1* promoter. **(G)** Purified Iml1-His$_6$ preferentially binds to bacterially expressed GST-Gtr1 preloaded with GTPγS, GTP, or AlF$_x$ (AlF$_3$ or AlF$_4^-$) plus GDP. Data from (E) to (G) are representative immunoblots from three or more independent experiments.

d) Iml1 is a GAP for Gtr1

To verify our assumption that Iml1 has GAP activity towards Gtr1, we performed *in vitro* GAP assays with purified Iml1 and Gtr1 proteins. In a concentration-dependent manner, Iml1 stimulated the rate of GTP hydrolysis by Gtr1 to a greater extent than that by Gtr2 or the unrelated Rho GTPase Cdc42 (Fig. 1.6A and B).

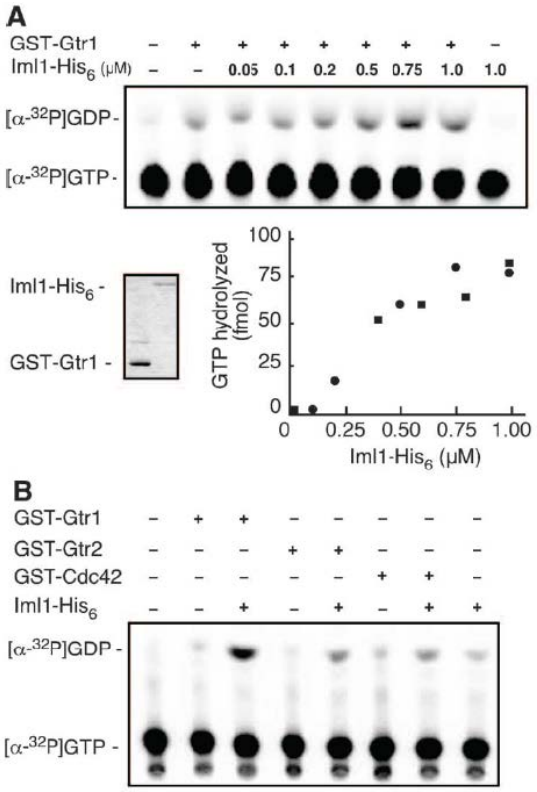

Fig. 1.6. Iml1 is a GAP for Gtr1. (A) GST-Gtr1 was loaded with [α -^{32}P]-GTP and hydrolysis to [α-^{32}P]-GDP was assayed in the absence or presence of increasing concentrations of Iml1-His$_6$. Purified proteins were visualized by Coomassie staining (inserted panel) and the results of the GAP assay were quantified and illustrated graphically. One representative TLC autoradiograph and the corresponding quantifications from two independent experiments (squares or circles) are shown. **(B)** Iml1-His$_6$ activates the intrinsic GTPase activity of Gtr1 to a greater extent than that of Gtr2 or the Rho GTPase Cdc42. One representative TLC autoradiograph from two independent experiments is shown.

Analyses of truncated Iml1 versions further allowed us to specify a conserved domain in Iml1 that was required for proper TORC1 inhibition (Fig. 1.7A) and was sufficient to stimulate the GTP hydrolysis rate by Gtr1 *in vitro* (Fig. 1.7B). In single turnover GAP assays, this domain, like full length Iml1, substantially accelerated the catalytic rate of Gtr1-mediated GTP hydrolysis and was therefore coined the Iml1 GAP domain (Iml1^{GAP}; Fig. 1.7A and C). GAPs often supply a catalytic amino acid residue such as an arginine (Arg), glutamine (Gln) or aspartate (Asp) into the active site of their

GTPases (Wittinghofer and Vetter, 2011), which prompted us to carry out an alanine scanning approach. We identified Arg^{943}, which is located within Iml1^{GAP} (Fig. 1.7A), as critical for Iml1 GAP activity *in vitro* (Fig. 1.7B and C). The R943A mutation significantly reduced the TORC1 inhibitory function of Iml1 in cells (Fig. 1.4E, 1.7D, and E). Together with the observation that GDP·AlF$_x$ normally promoted the Gtr1-Iml1^{R943A}-His$_6$ interaction (Fig. 1.7F), these data suggest that Arg^{943} is a catalytically, rather than structurally, important residue within the Gtr1 GAP Iml1. However, detailed structural analyses will be required to assess whether Arg^{943} in Iml1 interacts with the catalytic domain of Gtr1.

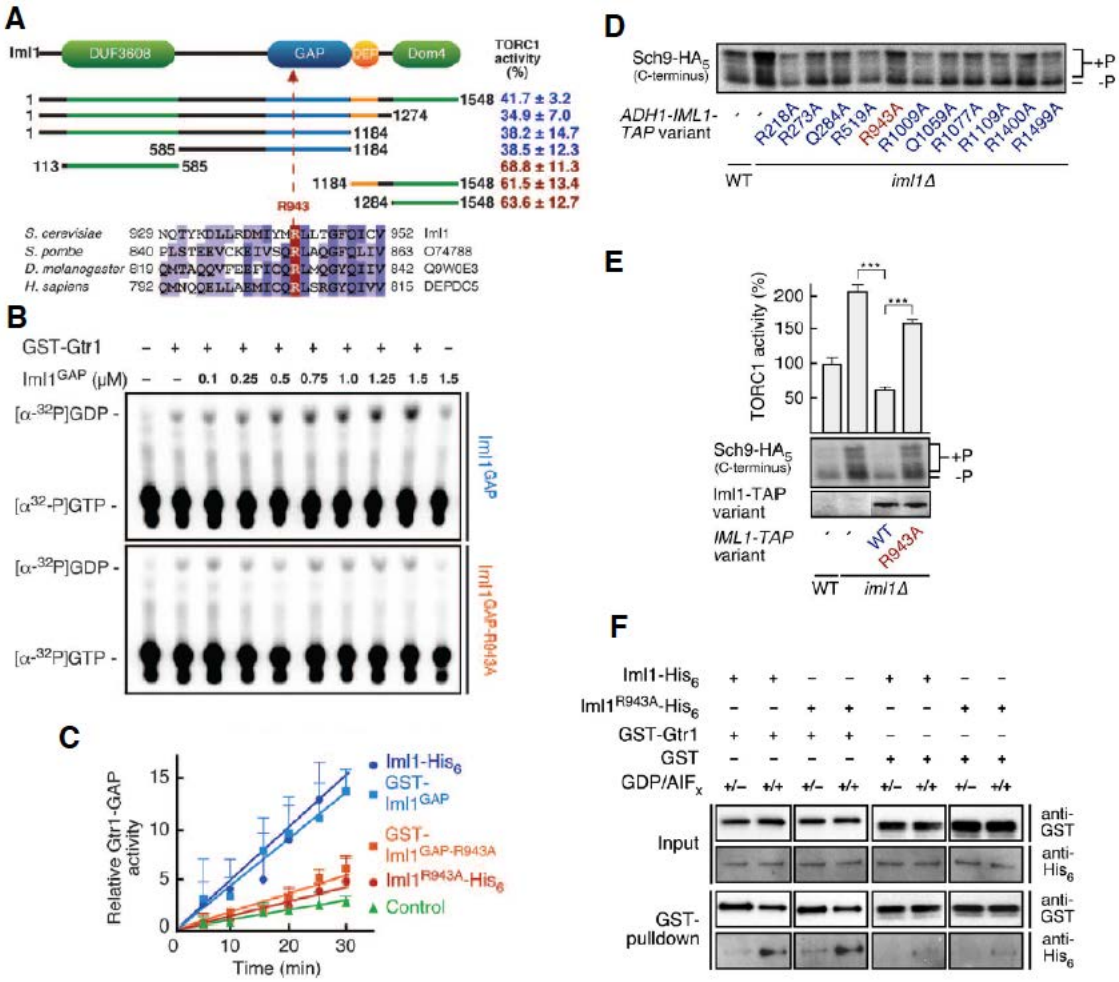

Fig. 1.7 Identification of Iml1 GAP domain (A) Schematic representation of the conserved domains within *S. cerevisiae* Iml1 and functional analysis of the TORC1 inhibitory activities of the indicated truncated Iml1 variants. The corresponding constructs were overexpressed in wild-type cells and TORC1 activities were normalized to the samples containing the empty vector. The red arrow indicates the position of a conserved arginine within the Iml1 GAP domain that was aligned, together with its flanking residues, with the corresponding amino acid sequences of Iml1 orthologs in higher eukaryotes. Data are means ± S.D. of three independent experiments. **(B)** The bacterially expressed GAP domain of Iml1 (Iml1^{GAP}), but not Iml1$^{GAP-R943A}$, activates the GTPase activity of Gtr1. One representative TLC autoradiograph from three independent experiments is shown. **(C)** Single turnover GAP assays on Gtr1 with or without (control) the indicated full-length Iml1 or Iml1^{GAP} variants. Data are means + S.D. from three independent experiments. **(D)** Alanine scanning of conserved arginine and glutamine residues in Iml1 indicates that Arg^{943} is important for the TORC1-inhibitory activity of overexpressed Iml1-TAP (assayed as in Fig. 1A). **(E)** Effects of overexpression of Iml1-TAP and Iml1^{R943A}-TAP on TORC1 activity

were assayed in three independent replicates (means + S.D. are shown). Fusion protein expression was confirmed by immunoblot analysis (lower panel). **(F)** Purified Iml1-His$_6$ and Iml1^{R943A}-His$_6$ preferentially bind bacterially expressed GST-Gtr1 preloaded with GDP plus AlF$_x$. *In vitro* GST-pulldown experiments were performed with bacterially expressed GST-Gtr1 preloaded with GDP alone (+/-) or GDP plus AlF$_x$ (+/+) and either purified Iml1-His$_6$ or Iml1^{R943A}-His$_6$. One representative Western blot from two independent experiments is shown ***P < 0.001, in Student's t-test (P-values are Holm-Bonferroni adjusted).

Gtr1 has a very low intrinsic activity with a k_{cat} of 0.004 min^{-1} compared to other GTPases, *i.e.* k_{cat} of G_{ial} = 3 min^{-1}, Ras = 0.3 min^{-1} and EF-TU = 0.003 min^{-1} (Sengottaiyan et al., 2012). We obtained a similar k_{cat} of 0.0023 min^{-1} for Gtr1 by classical Michaelis-Menten titration (Fig 1.8A). However, Gtr1-Gtr2^{Q66L} or Gtr1-Gtr2^{S23L} heterodimer formation *per se* stimulated the intrinsic GTPase activity of Gtr1 16- or 128-fold, respectively. Of note, Iml1 exhibited GAP activity towards both monomeric or heterodimeric Gtr1 in a comparable range (Fig. 1.8C and D). Thus, Gtr1-mediated GTP hydrolysis in Gtr GTPase heterodimers appears to be controlled synergistically by both the GDP/GTP loading status of Gtr2 and the activity of Iml1.

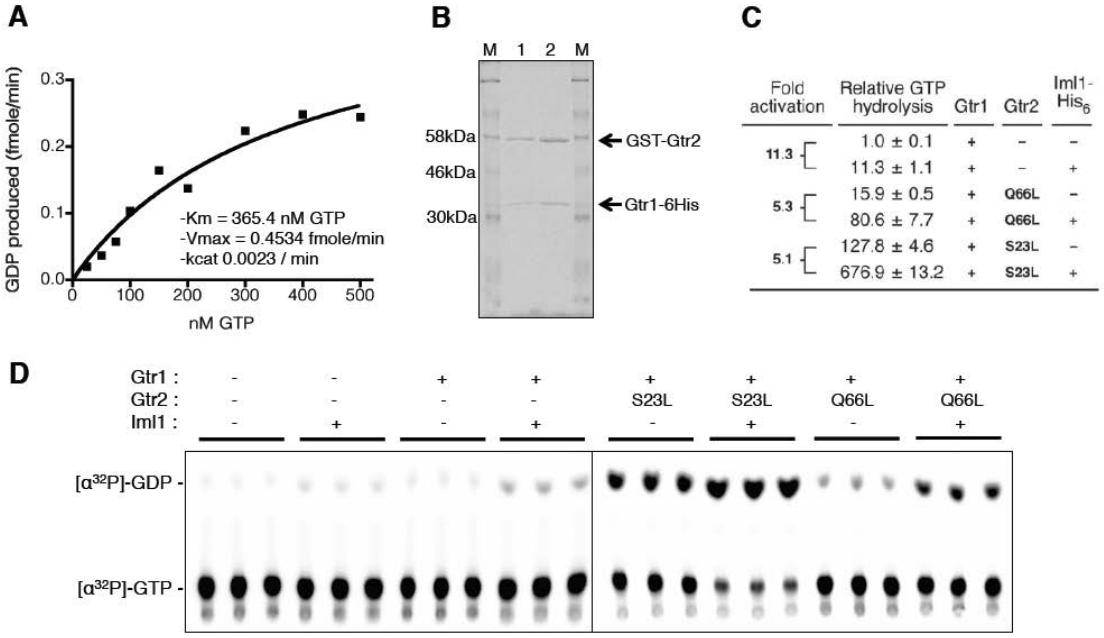

Fig 1.8 Iml1 and Gtr2 synergistically activate Gtr1 GTP hydrolysis. (A) Michaelis-Menten tritration on 10nM of purified GST-Gtr1 allowed determining Gtr1 K_m, V_{max} and K_{cat}. **(B)** Bacterially expressed Gtr1-6His and GST-Gtr1^{S23L} (line1) or GST-Gtr1^{Q66L} (line2) were co-purified by GST pulldown and visualized by coomassie staining. **(C)** Relative GTP hydrolysis (± S.D.; n=3 independent experiments) by the indicated combinations of Gtr GTPases (with or without Iml1-His$_6$; normalized to the one of Gtr1 without Iml1-His$_6$). **(D)** TLC autoradiograph used for quantification (C).

e) Expression of human *DEPDC5* partially complements loss of *IML1*

Iml1, Npr2, and Npr3 have orthologs in human (DEPDC5, NPRL2, and NPRL3, respectively), of which NPRL2 has previously been classified as a suppressor of various tumors (Ji et al., 2002; Lerman and Minna, 2000; Li et al., 2004). In addition, the identification of overlapping homozygous deletions encompassing *DEPDC5* (and two other genes) in two cases of glioblastoma suggests that loss of DEPDC5 may contribute to the development of cancer (Seng et al., 2005). Because human DEPDC5 was able to partially complement the TORC1 inhibition defect in *iml1Δ* yeast cells (Fig. 1.9), we speculate that SEACIT may play an evolutionarily conserved role in suppressing Rag-mediated activation of TORC1, thereby contributing to the suppression of human tumor formation.

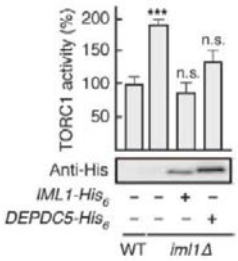

Fig. 1.9 Expression of human *DEPDC5* partially complements the TORC1 inhibition defect in *iml1Δ* cells. TORC1 activities were assayed as in Fig. 1.1C. Fusion protein expression was confirmed by immunoblot analysis. ***$P <$ 0.001, n.s., not significant, in Student's *t*-test compared to the respective wild-type control (*P*-values are Holm-Bonferroni adjusted). Data are means + S.D. from three independent experiments.

f) loss of Iml1 prevents DHBB-induced inactivation of TORC1

In a previous study, our group reported that the leucyl-tRNA synthetase mediates a leucine signal to TORC1 via the interaction of its CP1 editing domain with Gtr1 (Bonfils et al., 2012). This model proposed that the CP1 domain could prevent a negative regulator, such as a GAP, from having access to Gtr1. Leucine deprivation trigger a conformational change in CP1 to edit mischarged $tRNA^{Leu}$. This change disrupts the Cdc60-Gtr1 interaction, allowing GTP hydrolysis by Gtr1 and TORC1 inactivation. Similarly, treatment with 1,3-dihydro-1-hydroxy-2,1-benzoxa-borole (DHBB), which blocks CP1 in its editing conformation, disrupt Cdc60-Gtr1 interaction and results in TORC1 inactivation. Therefore we reasoned that the absence of the negative regulator should protect TORC1 from inhibition after DHBB treatment. Indeed, loss of Iml1 conferred DHBB resistance to TORC1 to a similar extent as expression of the Rag's signaling competent $Gtr1^{Q65L}$-$Gtr2^{S23L}$ form (Fig. 1.10A). However, in CoIP experiments DHBB treatment did not affect the Iml1-Gtr1 interaction and overexpression of Iml1 did not decrease the interaction between

Cdc60 and Gtr1 (Marie-Pierre Péli-Gulli, personal communication). Moreover, addition of purified recombinant CP1 did not inhibit Iml1 GAP activity *in vitro* (Fig. 1.10B). Thus, although genetic experiments indicate that Cdc60 and Iml1 affect TORC1 activity via the same branch, the mechanism by which Cdc60 regulates Gtr1 seems to be more complex than first thought and should be investigated in future experiments.

Alternatively, it has been proposed that the human ortholog of Cdc60, LARS1, activates TORC1 by stimulating GTP hydrolysis of the Gtr2 ortholog RagD (Han et al., 2012). However, a second group failed to show any GAP activity of LARS1 on RagC, the other ortholog of Gtr2 (Bar-Peled et al., 2013).

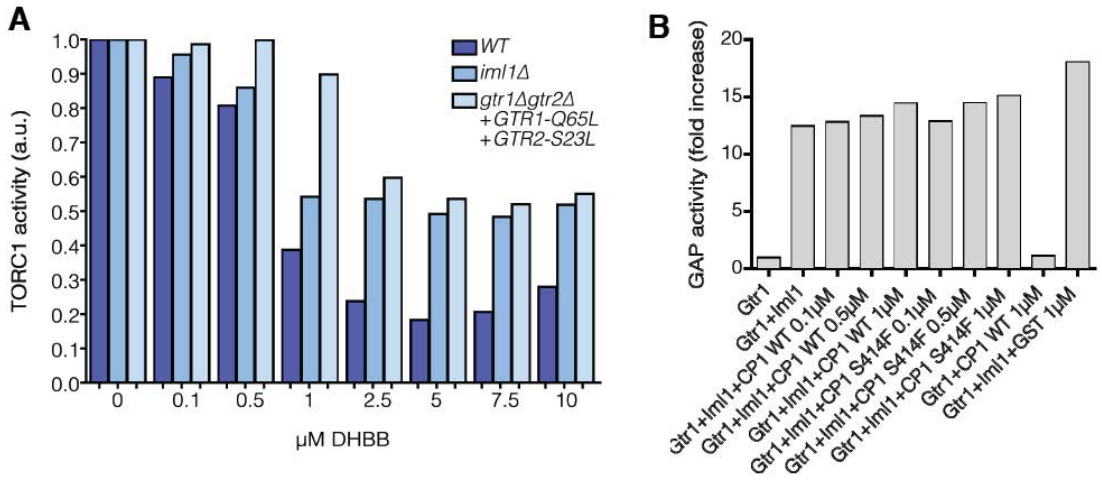

Fig 1.10. Relation between Iml1 and Cdc60. (A) *IML1* deletion partially protect cells from DHBB treatment, similarly to expression of signaling competent $Gtr1^{Q65L}$-$Gtr2^{S23L}$. TORC1 activities were assayed as in Fig. 1.1C. **(B)** CP1 domain doesn't inhibit Iml1 GAP activity *in vitro*. Iml1 GAP activity was assayed as in Fig. 1.6A in presence of an increasing concentration of recombinant GST-CP1 domain, control GST-$CP1^{S414F}$ domain (mutant defective in binding Gtr1) or GST alone.

g) SEACAT regulates SEACIT stability

Sea2, Sea3 and Sea4 contain RING domains that are commonly found in E3 ligases (Dokudovskaya et al., 2011). Furthermore, Npr2 has been shown to interact with the SCF^{GRR1} E3 ubiquitin ligase subunit Grr1 (Spielewoy et al., 2010). As negative regulator of SEACIT, we hypothesized that SEACAT might affect the stability of SEACIT components in response to changes in amino acid levels. In a WT context, the level of Npr3 protein is slightly decreased after 30 minutes of leucine starvation, while Iml1 and Npr2 remain stable (Fig. 1.11). To our surprise, in a strain lacking Sea2, Sea3 and Sea4, the levels of Iml1 and Npr3 are constantly low in the presence

or absence of leucine. This result argues against a model where Sea2-Sea3-Sea4 acts as an E3 ubiquitin ligase and promotes degradation of SEACIT components. Interestingly, we observed a band-shift for Npr2 that disappears in absence of Sea2, Sea3, and Sea4 (Fig. 1.11). It has been previously reported that Npr2 is phosphorylated in a Yck1/2 dependent manner (Spielewoy et al., 2010) and that Iml1 preferentially interacts with phosphorylated Npr2 (Wu and Tu, 2011). Thus, although Sea2-Sea3-Sea4 appear to promote the stability of Iml1 and Npr3, it is likely that SEACAT plays an important role in the phospho-regulation of Npr2. Further experiments are required to decipher the exact mechanism by which SEACAT negatively regulates SEACIT.

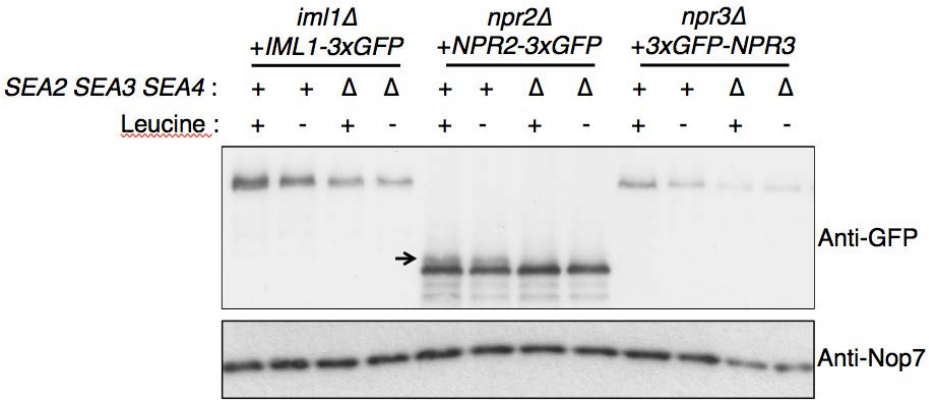

Fig. 1.11. Iml1-GFP and Npr3-GFP levels are impaired in *sea2Δsea3Δsea4Δ* mutant. The total protein levels of Iml1-GFP_3, Npr2-GFP_3, and GFP_3-Npr3 were probed (via immunoblot analysis) in cells with the indicated background that were grown in the presence (+) or absence (-; 30 min starvation) of leucine. The arrow indicates band-shift in Npr2-3xGFP migration. The pre-ribosomal particle component Nop7 was used as loading control.

1.3 Discussion

Recent studies implicated subunits of the octameric vacuolar Seh1-associated Complex (SEAC) in negative regulation of TORC1 in yeast (Dokudovskaya and Rout, 2011; Dokudovskaya et al., 2011; Neklesa and Davis, 2009; Wu and Tu, 2011). In an effort to clarify the relationship between SEAC and TORC1, we discovered in genetic epistasis analyses that the Iml1-Npr2-Npr3 SEAC subcomplex, which we now name SEACIT (for SEAC subcomplex Inhibiting TORC1 signaling), negatively regulates TORC1 through Gtr1 within the EGOC. Moreover, in line with our genetic data, we found that leucine deprivation triggered Iml1 to transiently interact with Gtr1 (in a Npr2- and Npr3-dependent manner) to stimulate its intrinsic GTPase activity. Of note, both Npr2 and Npr3 contain a N-terminal longin domain, the structure of which is closely related to Roadblock domains and may serve as platform for Rag GTPases (Levine et al., 2013). A report published in parallel to ours showed that the GAP activity of SEACIT is conserved, as the orthologous complex in *Drosophila* and human cells (i.e. DEPDC5-Nprl2-Nprl3) coined GATOR1 also acts as a GAP toward RagA and RagB (Bar-Peled et al., 2013). Intriguingly, various glioblastomas and ovarian cancers contain nonsense or frameshift mutations or truncating deletions in GATOR1-encoding genes and a number of cancer cell lines with homozygous deletions in *DEPDC5*, *NPRL2*, or *NPRL3* exhibit hyperactive mTORC1 that is insensitive to amino acid deprivation (Bar-Peled et al., 2013). Since these GATOR1-inactivating mutations also cause hypersensitivity to the TORC1 inhibitor rapamycin in mammalian cells, they may help to predict the therapeutic benefit of clinically approved TORC1 inhibitors in cancer treatments (Bar-Peled et al., 2013).

In addition to Iml1, Npr2, and Npr3 (SEACIT), the octameric SEAC also contains Sea2, Sea3, Sea4, Seh1, and Sec13, orthologs of the mammalian and *Drosophila* GATOR2 subcomplex proteins WDR24, WDR59, Mios, Seh1L, and Sec13, respectively. These proteins form the other SEAC-subcomplex, which we now name SEACAT (for SEAC subcomplex Activating TORC1 signaling). Except for Sec13, all of the GATOR2 components have been implicated in negative regulation of GATOR1 in higher eukaryotes (Bar-Peled et al., 2013). Similarly, yeast SEACAT (including Sec13) antagonize the SEACIT-mediated TORC1 inhibition. We also show that SEACAT components are implied in SEACIT stability and possibly in Npr2

phosphoregulation. These data therefore support a model in which SEACAT promotes TORC1 activity through inhibition of the GAP-function of SEACIT. These results extend the remarkable evolutionary conservation of TORC1 regulation by Rag GTPases and delineate an inhibitory role for the pentameric SEACAT/GATOR2 subcomplex upstream of the SEACIT/GATOR1 subcomplex (Fig. 1.12).

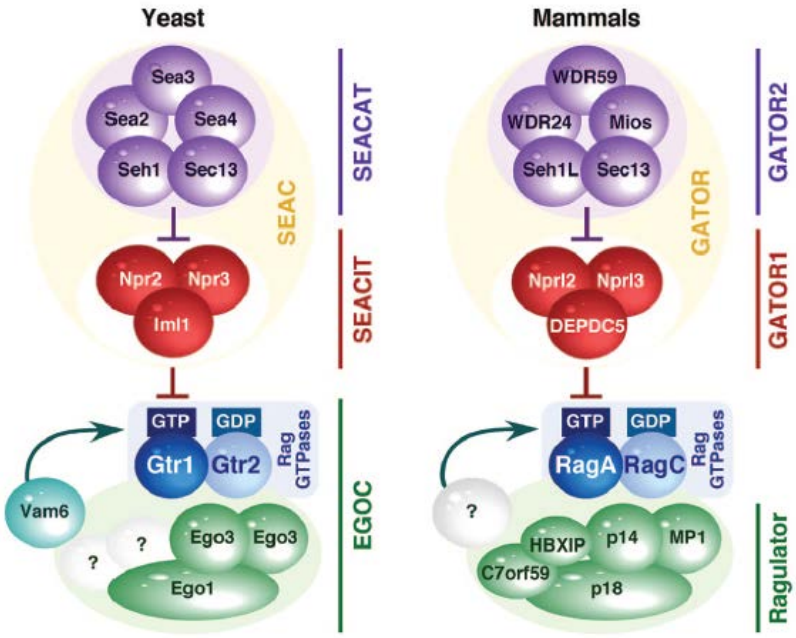

Fig. 1.12. Conserved regulators of the Rag-family GTPases. The yeast SEAC is composed of 2 subcomplexes, SEACIT and SEACAT. SEACAT antagonizes the GAP-function of SEACIT. Vam6 is thought to be the GEF for Gtr1, which resides in the EGOC on the vacuolar membrane. Similarly, the mammalian (and *Drosophila*) GATOR complex is composed of the 2 subcomplexes GATOR1 and GATOR2. GATOR2 antagonizes the GAP-function of GATOR1. Whether or not mammalian Vam6 orthologs (i.e., the TGF-β Receptor Associated Protein-1 [TRAP1 or TGFBRAP1] and the TRAP1-Like-Protein [TLP], aka hVPS39) act as a RagA/B GEF is unclear, rather the pentameric Ragulator complex, acting downstream of the vacuolar ATPase, is reported to serve this function. For details, please see text.

Chapter II :

Ego2 is a new member of the EGO complex that is essential for TORC1 activation

2.1 Introduction

Mammalian pentameric Ragulator, composed of p18, Mp1, p14, HBXIP and C7orf59, has GEF activity toward RagA/B (Bar-Peled et al., 2012). So far in yeast, such a function has not been shown for the EGOC and orthologs of HBXIP and C7orf59 have not been identified. A recent study used HHpred, a highly sensitive method for homology detection, to identify two uncharacterized yeast proteins known as Ycr075w and Ynr034w as potential HBXIP and C7orf59 homologs (Fig.2.1) (Levine et al., 2013). Interestingly, the Ynr034w NMR structure harbors a Roadblock-like fold (available in PDB as 2GRG) (Levine et al., 2013). Here we will investigate the role of Ycr075w and Ynr034w as potential HBXIP and C7orf59 homologs and their implication in TORC1 signaling. For convenience and reasons that will be discussed later in this chapter, we have renamed Ycr075w as Ego2 and Ynr034w as Ego4.

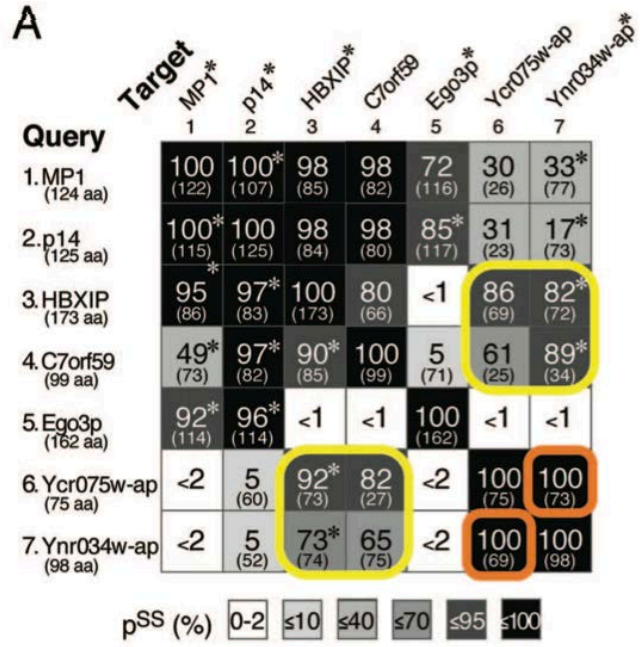

Fig.2.1 Structural homology between known Ragulator members and new yeast Roadblock containing protein. The probabilities of shared structure (p^{ss}) obtained by HHpred and the number of aligned residues (in brackets) are indicated. Yellow boxes highlight that Ycr075w and Ynr034w are closely related to HBXIP and C7orf59. Orange boxes show that Ycr075w and Ynr034w are highly related to each other. Taken from (Levine et al., 2013).

2.2 Results

a) Ego2 but not Ego4 is necessary for TORC1 activity.

To investigate the possible link between Ego2, Ego4 and TORC1 we first assayed the phosphorylation of the direct TORC1 target Sch9 in cells lacking Ego2 and/or Ego4. In the absence of Ego2, but not Ego4, TORC1 activity was decreased (Fig.2.2.A and B). Because Ego2 and Ego4 are highly related to each other it remained a possibility that in the absence of Ego4, Ego2 may be able to assume its function. Deletion of both *EGO2* and *EGO4* resulted in a slightly stronger impairment of TORC1 activity than the single *EGO2* deletion (Fig.2.2.A and B). In contrast with the drastic effect of its loss, *EGO2* overexpression did not affect TORC1 activity. The same result was observed after *EGO4* overexpression and the combined overexpression of *EGO2* and *EGO4* (Fig.2.2.C).

We next tested if Ego2 affected TORC1 activity through the EGOC. To this end we combined the deletion of *EGO2* with expression of different Gtr1 and Gtr2 alleles. TORC1 activity was constantly low in cells expressing signaling-compromised $Gtr1^{S20L}$ or $Gtr2^{Q66L}$ alleles, independently of the presence or absence of Ego2 (Fig.2.2.A and B). However, expression of either one of the signaling proficient forms of Gtr1 or Gtr2 (*i.e.* $Gtr1^{Q65L}$ or $Gtr2^{S23L}$) failed to cause TORC1 hyperactivation in cells lacking Ego2, but were still able to rescue TORC1 activity in these cells to WT levels (Fig.2.2.A and B). From these results, we infer that either Ego2 signals to TORC1 in parallel to EGOC, or Ego2 may be part of the EGOC and in its absence the complex is destabilized, resulting in impaired ability to activate TORC1.

The loss of Ego4 did not impair the effects of the different Gtr1 or Gtr2 forms on TORC1 activity, except in the case of $Gtr2^{S23L}$. Surprisingly, cells lacking Ego4 and expressing $Gtr2^{S23L}$ had low TORC1 activity (Fig.2.2.A and B) and increased sensitivity to rapamycin (Katie Powis, personal communication). This synthetic sick interaction is the only phenotype linked to *EGO4* so far.

Taken together these results show that Ego2 is required for TORC1 activity and could act either in parallel of or as component of the EGOC. Although the role of Ego4 is more obscure, it appears to be genetically linked to Gtr2.

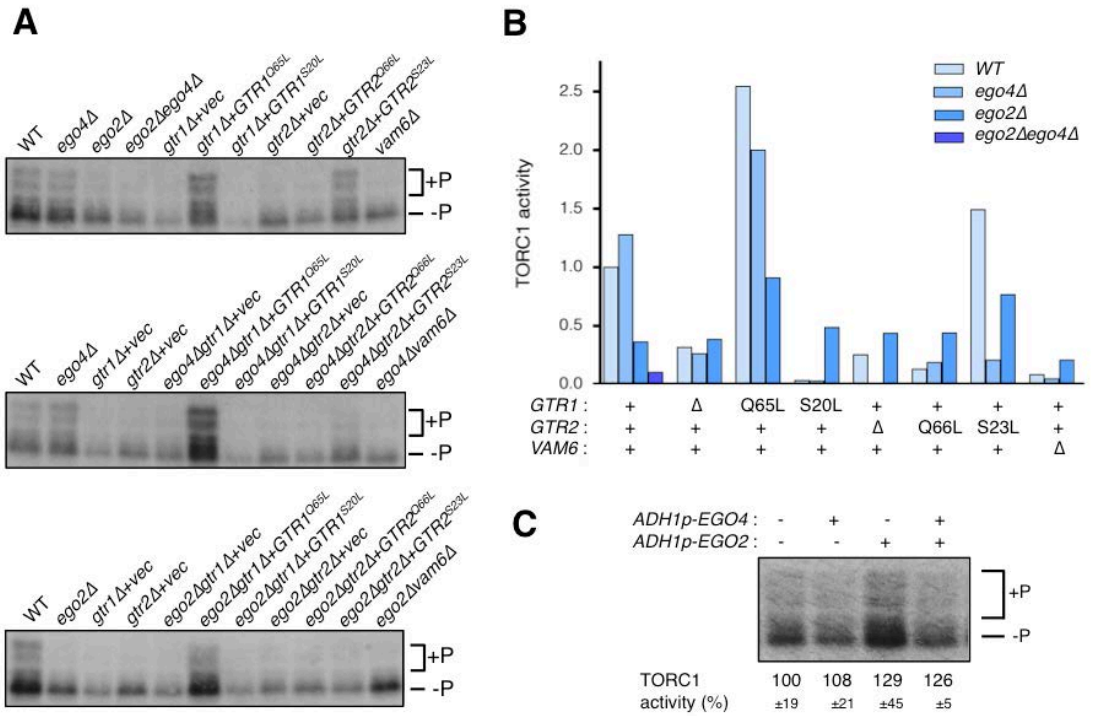

Fig.2.2 Ego2, but not Ego4, is required for proper TORC1 activity. (**A, B**) TORC1 activity in cells lacking Ego2 or Ego4 individually or in combination with different *GTR1/2* alleles. (A) Imunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 were used to quantify TORC1 activity (the ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9) (Urban et al., 2007). (B) The values were normalized to the ones for wild-type (WT) cells and presented in the bar graph. One representative immunoblot from at least two independent experiments is shown. (**C**) Overexpression of Ego2 and/or Ego4 does not affect TORC1 activity. Sch9 phosphorylation was assessed as in (A) and normalized as in (B). Values indicate means ± SD of three independent experiments.

b) Ego2 is required for EGOC integrity.

EGOC localization at the vacuolar rim is required for its proper function in TORC1 activation. The vacuolar localization of Gtr1 and Gtr2 is dependent on Ego3, and Ego1 is necessary to anchor the entire complex at the vacuolar membrane (Zhang et al., 2012). To investigate if Ego2 is involved in EGOC assembly we analyzed the localization of Gtr1 and Gtr2. Both proteins required Ego2, but not Ego4, to properly localize at the vacuolar periphery (Fig.2.3). Hence it seems that Ego2 acts directly on the integrity of the EGOC rather than in parallel to it.

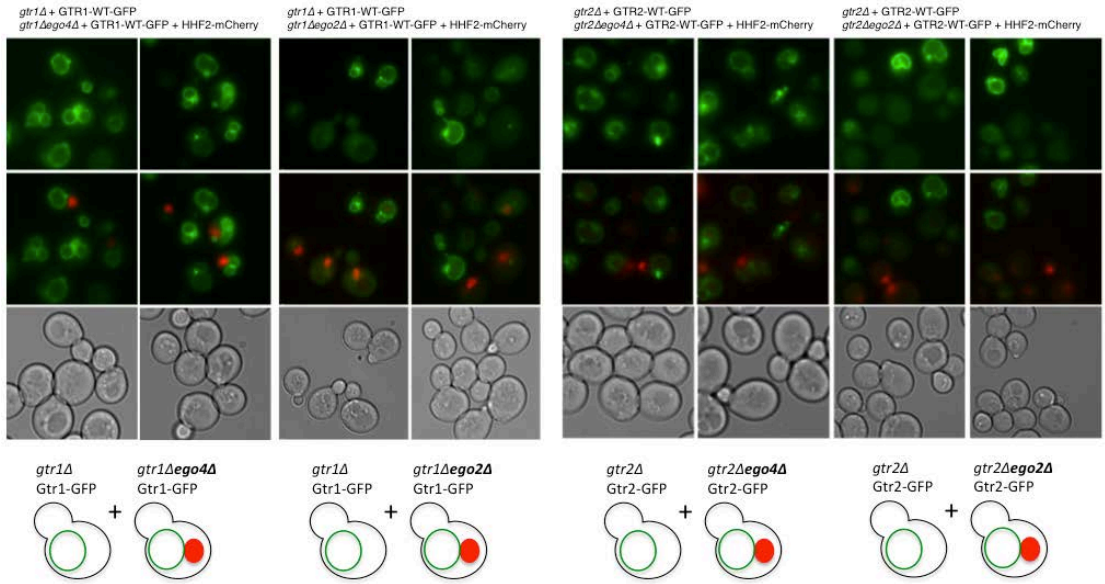

Fig.2.3 Gtr1 and Gtr2 require Ego2, but not Ego4, to localize at the vacuolar membrane. Strains expressing the indicated functional GFP-fusion Rag GTPase from its own promoter in the absence or presence of Ego2 or Ego4 were analyzed by fluorescence microscopy during exponential growth. Cells lacking either Ego2 or Ego4 also expressed the red nuclear marker Hhf2-RFP and were mixed with cells harboring wild-type Ego2 or Ego4 to directly compare Gtr1 or Gtr2 signals at the vacuolar membrane. Images taken by Katie Powis.

c) Ego2 is part of the EGOC

Next, we attempted to determine the localization of Ego2 and Ego4 themselves. Unfortunately, we were unable to obtain a functional GFP tagged version of either protein. However, Ego2 C-terminally fused to GFP localized at the vacuolar periphery and in discrete dots linked to the vacuole, reminiscent of the localization of EGOC components (Fig.2.4.A) (Binda et al., 2009). Ego4-GFP displayed a diffuse cytoplasmic localization pattern with a slight accumulation in the nucleus (Fig.2.4.A).
The vacuolar localization of Ego2-GFP raised the possibility that Ego2 physically interacts with the EGOC. Indeed, in the split ubiquitin based two-hybrid system, Ego2 interacts strongly with Ego1 and to a lesser extent with Gtr1-GTP and Ego3 (Fig.2.4.B). Surprisingly, Ego4 interacts with Gtr1 (WT and GTP-bound forms) and with Gtr2-WT. In this system, the bait protein (Ego2 or Ego4) is artificially localized to membranes. As Ego2 and Ego4 are predicted to have highly similar structures (Levine et al., 2013), it is possible that Ego4 interacts with Gtr's only when it is forced to localize to membranes, but not necessarily under physiological conditions. To address this possibility we assessed the interaction of Gtr1 as membrane-bound bait, and Ego4 (or Ego2) as soluble prey. Unfortunately, both Ego4 and Ego2 showed

unspecific interactions with unrelated proteins when used as preys (data not shown). For the same reason we could not use this system to test if Ego2 and Ego4 form a dimer as predicted by Levine *et al*.

To further analyze the nature of Ego2 and Ego4 interactions with the EGOC, we co-expressed codon optimized versions of *EGO2, EGO3* and *EGO4* together with 6His N-terminally tagged *EGO1* in bacteria, and performed nickel-affinity purifications followed by size exclusion chromatography. Ego2 and Ego3, but not Ego4, co-purified in one complex with recombinant Ego1 (Fig.2.4.C and D). Finally, we tested the GEF activity of the bacterially purified complex containing 6His-Ego1, Ego2 and Ego3 toward Gtr1 *in vitro* and found that addition of purified EGOC did not increase the Gtr1 nucleotide exchange rate (data not shown). In sum, we show that Ego2 localizes at the vacuolar rim where it physically associates with EGOC through direct interactions with Ego1 and Ego3, strongly suggesting that Ego2 is in fact a subunit of the EGOC that has hitherto escaped detection.

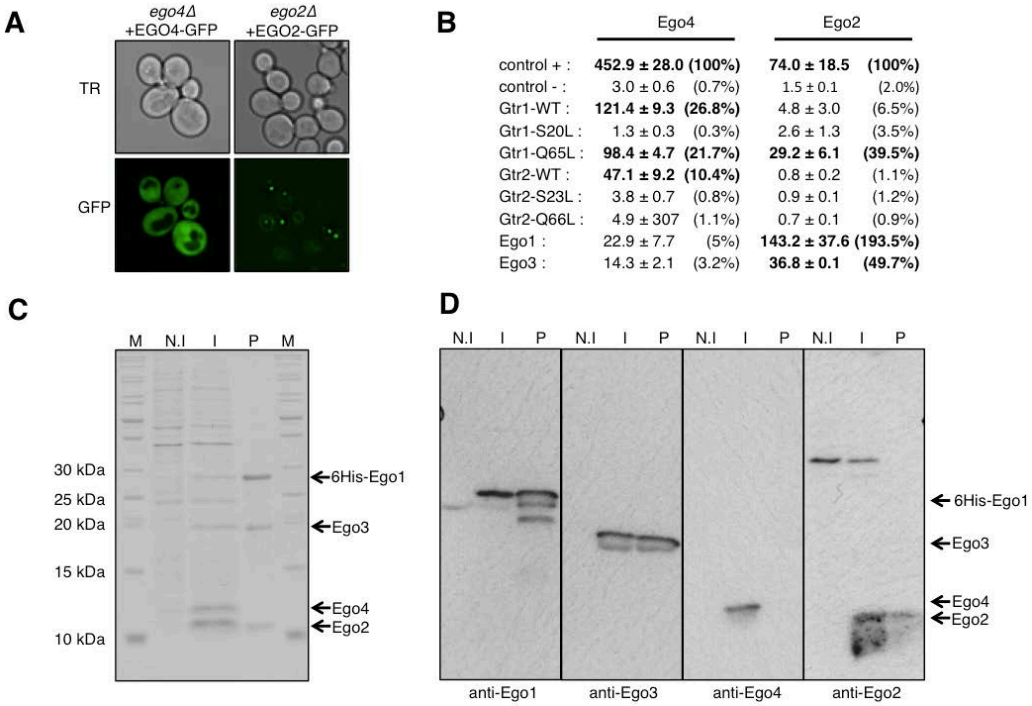

Fig.2.4 Ego2 directly interacts with the EGOC. (**A**) Strains expressing the indicated GFP-fusion proteins from their own promoter were analyzed by fluorescence microscopy during exponential growth. Images taken by Katie Powis. (**B**) The split ubiquitin-based membrane two-hybrid system (Dualsystems Biotech) was used to test the interaction of Ego2 or Ego4 (baits) with EGOC components or control proteins (Prey). For each combination tested, β-galactosidase activity is expressed in Miller units as mean ± SD from at least three independent transformants. Numbers in brackets show values normalized on the respective positive controls and expressed as percentage of activity. Bold numbers indicate an activity more than 10 fold higher than negative controls. (**C, D**) 6His-Ego1, Ego2, Ego3 and Ego4 were expressed in bacteria and subsequently purified using a nickel affinity column followed by size exclusion chromatography. Samples were taken at different steps and analyzed by SDS page followed by (C) Coomassie staining or (D) immunoblot using antibodies raised against the indicated protein. M denotes Marker, N.I. for not induced sample, I for IPTG induced sample and P for purified sample.

2.3 Discussion

Here we have characterized Ycr075w as a new subunit of the EGOC that is essential for proper assembly of the complex and subsequent TORC1 activation. For these reasons we have renamed Ycr075w to Ego2. A role for the Ego2 paralog Ynr034w/Ego4 in TORC1 signaling is less clear. Although we found that Ego4 interacts with Gtr1 and Gtr2 in two-hybrid assays, it is possible that this interaction is an artifact arising from the high structural similarity of Ego4 and Ego5 and the two-hybrid system used that forces Ego4 to localize at membranes. Furthermore, Ego4 did not co-purify with other EGOC components when co-expressed in bacteria. Nevertheless, we cannot exclude that Ego4 may require another yet unidentified protein to interact with the EGOC. *In vivo* co-immunoprecipitation experiments may be able to clarify this point. Although cells either lacking or overexpressing Ego4 did not exhibit any alterations in TORC1 activity, loss of *EGO4* resulted in reduced TORC1 activity when combined with the $GTR2^{S23L}$ allele that typically causes TORC1 hyperactivation in wild-type cells. Screening for suppressors of the rapamycin sensitivity of an *ego4Δ* $GTR2^{S23L}$ strain could provide valuable insight into the nature of this genetic interaction.

Ego2 and Ego4 are predicted to dimerize and to be the orthologs of human Ragulator components HBXIP and C7orf59 (Levine et al., 2013). Whereas the formation of an HBXIP and C7orf59 heterodimer is required for their interaction with other Ragulator members (Bar-Peled et al., 2012), our data only identify Ego2 as an EGOC component. An interesting possibility is that Ego2 forms a homodimer that would be the equivalent of the HBXIP-C7orf59 heterodimer in a similar manner to the Ego3 homodimer that is the structural counterpart of p14/MP1 (Zhang et al., 2012). Unfortunately, for the reasons discussed previously, we were not able to determine if Ego2 is able to form heterodimers by our split-ubiquitin two-hybrid analysis. Co-IP experiments will be necessary to answer this question. Such experiments could also be conducted to reveal whether amino acid levels regulate interactions between EGOC complex members. In human cells, the interaction between Ragulator and Rag GTPases was first reported to be insensitive to amino acids (Sancak et al., 2010), but subsequent experiments showed that the absence of amino acids actually strengthens the resepctive interaction (Bar-Peled et al., 2012).

Although we were unable to obtain functional GFP tagged versions of Ego2 and Ego4, Ego2-GFP localized at vacuolar rim, whereas Ego4-GFP was diffused in

cytoplasm and nucleus. This result is consistent with the notion that Ego2, but not Ego4, is an EGOC component, but should be confirmed by immunofluorescence assay against endogenous or functional tagged versions of Ego2 and Ego4. With this technique we could also determine if Ego1 and/or Ego3 are required for the localization of Ego2 at the vacuolar periphery.

Our data support a role for Ego2 as a structural subunit of the EGOC. However, there is so far no indication that the expanded EGOC (Ego1-Ego2-Ego3) has GEF activity toward Gtr1: i) Ego2 (and Ego4) interact preferentially with GTP-bound Gtr1 in two hybrid assay, whereas GEFs typically bind more strongly to nucleotide free forms of their target GTPases; ii) Ego2 and/or Ego4 overexpression did not result in TORC1 hyperactivation; and iii) purified EGOC did not stimulate the nucleotide exchange activity of Gtr1 *in vitro*. Of course we cannot exclude that other yet unidentified components are required for GEF activity towards Gtr1, especially components providing a catalytic domain for the reaction. The Ego3 dimer adopts a Roadblock conformation that interacts with Gtr's and Ego2 is predicted to adopt the same conformation. Roadblock domains can stabilize GTPases and thus promote GTP hydrolysis (Miertzschke et al., 2011), but it is unlikely that they directly promote nucleotide exchange through destabilization of the G-domain. Ego1 does not contain any region resembling a known GEF domain and rather seems to act as a scaffold for the complex. Thus it seems that in yeast the EGOC assumes a structural rather than a catalytic function for Rag GTPases. Interestingly, in the previous chapter we demonstrated that EGOC is required for the localization of the Gtr1-GAP Iml1 at the vacuolar membrane; hence the EGOC could act as a general modulator of interactions between Gtrs and their regulators. It would be interesting to investigate the role of the expanded EGOC in the recruitment of the Gtr1-GEF Vam6 and on other regulators of the Gtr GTPases.

Chapter III :

Identification of new TORC1 inhibitors

Part of the work from this chapter has been published in
ACS Chemical Biology, vol. 7 (issue 4).

3.1 Introduction

The TOR pathway is deregulated in many human cancers. Specific inhibition of mTORC1 has emerged as a strategy for anticancer therapy (Zoncu et al., 2010). FDA approval of rapamycin as an immunosuppressant to prevent organ transplant rejection in 1999 accelerated investigations into its potential clinical use as an anticancer drug (Wander et al., 2011). Despite the crucial role of mTORC1 in cell homeostasis, rapamycin is well tolerated (Wander et al., 2011). However, the first generation of mTORC1 inhibitors (i.e. rapamycin and rapalogs) had limited success in cancer treatment. This is mainly due to the complex crosstalk between mTORC1, mTORC2 and PI3K that results in mTORC2 and PI3K activation after mTORC1 inhibition and the fact that rapamycin does not inhibit all mTORC1 functions (Benjamin et al., 2011). Currently, a second generation of inhibitors is undergoing clinically testing. These molecules target the kinase activity of mTOR and are able to block both mTORC1 and mTORC2 (Benjamin et al., 2011). A third class of compounds consists of dual PI3K/TOR kinase inhibitors. Both TOR and PI3K/TOR kinase inhibitors have demonstrated promising results in clinical trials (Wander et al., 2011). Despite considerable progress, it remains worth investigating potential new TOR inhibitors. Indeed, different manners of inhibiting TOR will allow a more gradual and specific response to tumors in a personalized medicine context. Furthermore, the use of novel TOR inhibitors in fundamental research may aid the discovery of TOR-regulated processes that are not blocked with rapamycin. Here I will discuss the effect of different molecules on TORC1 in yeast.

3.2 Results

a) CID 3528206 is a small molecule drug that inhibits TORC1

The new generation of kinase inhibitors very efficiently block mTOR functions but act both on mTORC1 and mTORC2. Small molecules that efficiently and selectively block either mTORC1 or mTORC2 are lacking. To identify such specific molecules for mTORC1, the group of Larry Sklar in New Mexico used a high-throughput flow cytometry approach on budding yeast. The expression levels of five GFP-tagged proteins (*CIT2*, *AGP1*, *MEP2*, *LAP4,* and *RPL19A*) that were known to be controlled by TORC1-regulated signalling branches were monitored in presence of a library of

compounds. Out of 320'000 compounds, 255 altered the expression of one or more GFP clones, but only one altered the expression of all five reporters (Chen et al., 2012). This compound, coined CID 3528206 (Fig.3.1A), inhibited growth in WT yeast cells, but not in a TORC1 bypass strain that expresses phosphomimetic $Sch9^{2D3E}$ and is deleted for *GLN3* (Bun-Ya et al., 1992; Chen et al., 2012; Gong et al., 2011; Urban et al., 2007; Wanke et al., 2008). To confirm that CID 3528206 acts on TORC1, we assessed *in vivo* Sch9 phosphorylation. CID 3528206 caused a dose dependent dephosphorylation of Sch9 (Fig.3.1B). Quantification of this result gave an IC_{50} of 3.9 μM (Fig.3.1C). Of note, CID 3528206 was able to inhibit TORC1 kinase activity *in vitro* with an IC_{50} of 150 nM, confirming that CID 3528206 directly targets TORC1 rather than acting on an upstream component (Chen et al., 2012). In line with these findings, microarray analysis showed that treatment of cells with rapamycin or with CID 3528206 resulted very similar changes in global gene expression profiles (*i.e.* 85.1% shared regulated genes) (Chen et al., 2012).

It would be interesting to now focus investigations on the genes that are regulated by CID 3528206 but not by rapamycin to identify new processes regulated by TORC1. Structural studies will also be necessary to understand the mechanism by which CID 3528206 inhibits TORC1. In adition future efforts to determine if the compound is also active on human mTORC1 will be of upmost importance. If this is the case, structure-activity relationship exploration may allow optimization of the compound for use as a therapeutic agent. In sum, the discovery of CID 3528206 as a novel TORC1 inhibitor may open the way for the development of novel TOR inhibitors with potential anticancer applications.

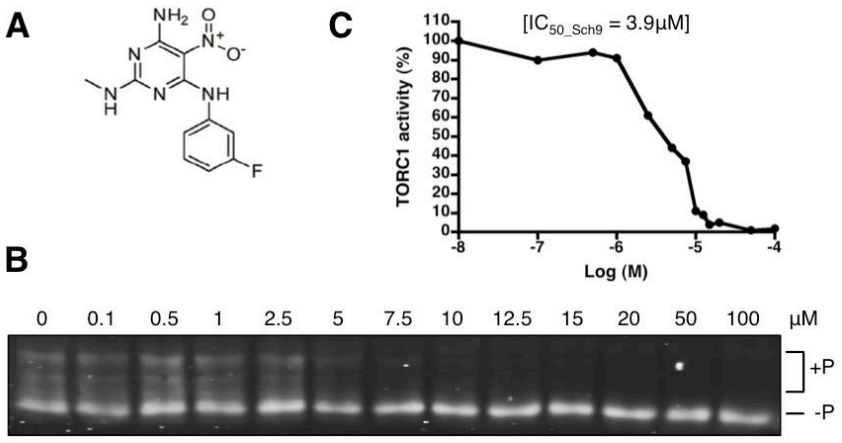

Fig.3.1. CID 3528206 inhibits TORC1 *in vivo*. (**A**) Chemical structure of CID 3528206. (**B, C**) Wild-type cells were treated with the indicated concentration of CID 3528206 for 30 min. (B) Immunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 (Urban et al., 2007). (C) the ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9 was used to quantify TORC1 activity. One representative immunoblot from two independent experiments is shown.

b) Polyamines inhibit TORC1

Polyamines are organic cations that are found in all organisms (Gerner and Meyskens, 2004). In eukaryotes, three polyamines, namely spermidine, spermine and their precursor putrescine are required for growth (Casero and Marton, 2007). Polyamines act at various levels to promote cell growth and have been shown to play critical roles in the maintenance of chromatin conformation, regulation of specific gene expression, ion-channel regulation, maintenance of membrane stability, hypusination of the eukaryotic translation initiation factor 5A (eIF5A), and free-radical scavenging (Casero and Marton, 2007). Polyamines can be imported from the environment or synthetized by the cell from the amino acid ornithine (Casero and Marton, 2007). The metabolism of arginine provides ornithine (through the urea cycle in mammals), which is then decarboxylated to give putrescine. Spermidine synthase uses putrescine to produce spermidine, which can be further processed to spermine through spermine synthase (Fig.3.2) (Casero and Marton, 2007). Interestingly, polyamine levels decrease in ageing mammalian and yeast cells, but are upregulated in cancer (Eisenberg et al., 2009; Scalabrino and Ferioli, 1984). Eisenberg *et al.* showed that addition of exogenous spermidine promotes longevity by inducing autophagy in yeast, fly, worm and human cells. They proposed that spermidine increases lifespan through inhibition of histone acetyltransferase activity (Eisenberg et al., 2009). Increased lifespan and induction of autophagy are hallmarks of TORC1 inhibition (Fontana et al., 2010). Thus we decided to investigate if the effect of spermidine on longevity could also be mediated through TORC1.

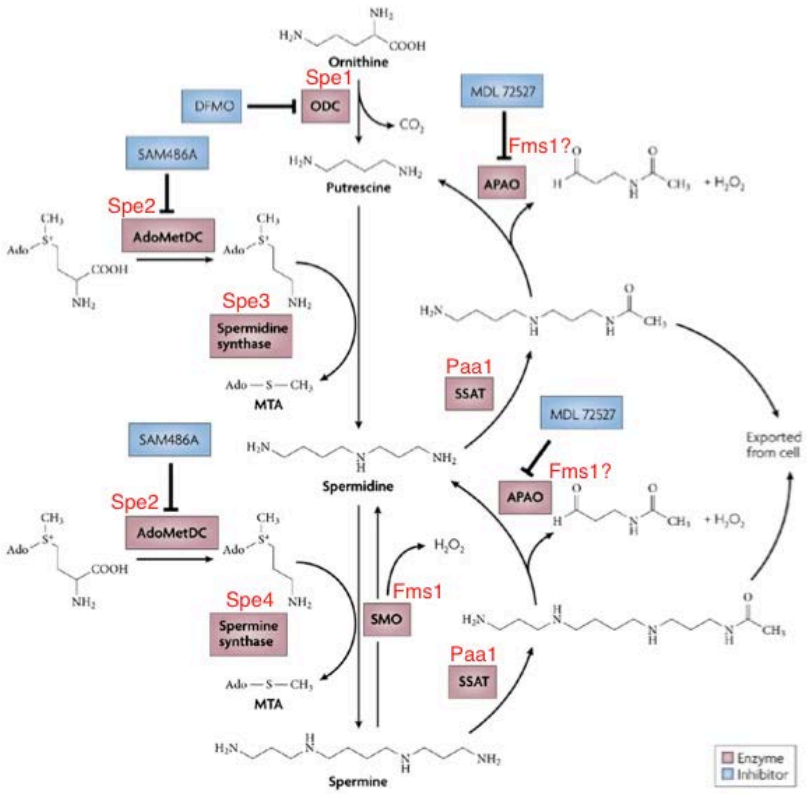

Fig.3.2 Metabolism of Polyamines. Human enzymes involved in polyamine metabolism are in pink boxes and their yeast counterparts are indicated in red. Blue boxes represent molecules targeting the pathway. For more detail see text. Adapted from (Casero and Marton, 2007).

After 30 minutes of treatment, both spermidine and spermine inhibit TORC1 activity *in vivo* in a dose dependent manner with an IC_{50} of 15.6 mM and 12.8 mM, respectively (Fig.3.3A, B and C). This is in a range which seems to be physiologically important as Eisengerg *et al.* observed increase in yeast lifespan and autophagy induction with concentrations of 4 mM of spermidine (Eisenberg et al., 2009).

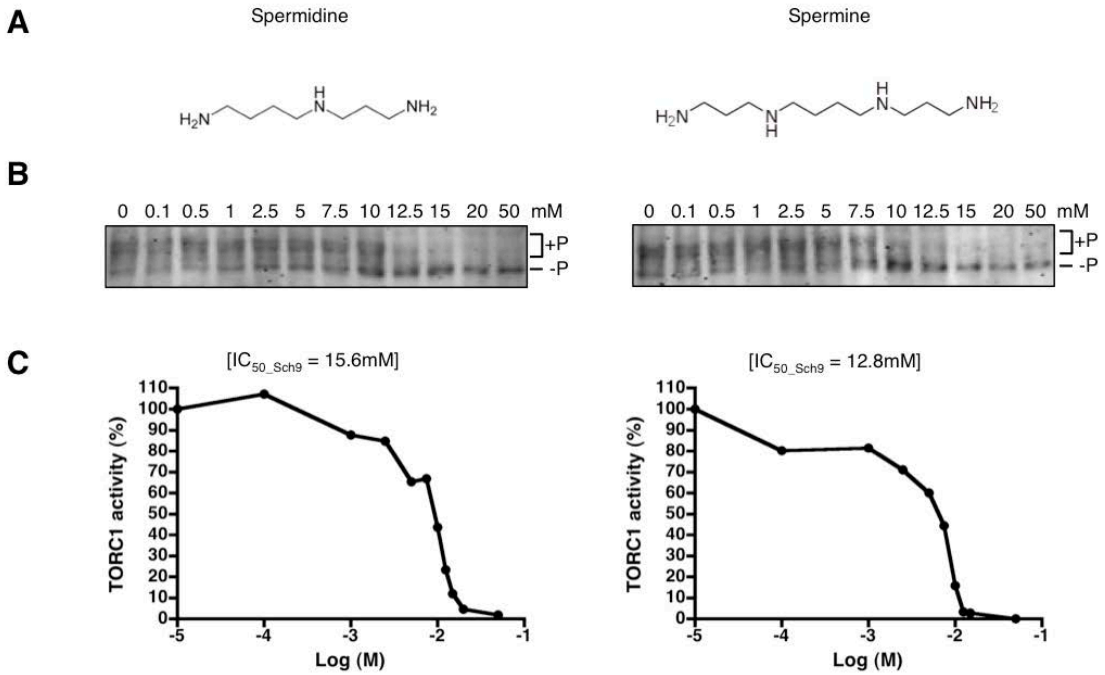

Fig.3.3. Polyamines inhibits TORC1 *in vivo*. (**A**) Chemical structure of spermidine and spermine. (**B, C**) Wild-type cells were treated with the indicated concentration of spermidine (left) or spermine (right) for 30min. (B) Immunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 (Urban et al., 2007). (C) The ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9 was used to quantify TORC1 activity.

These *in vivo* data do not allow the differentiation between a direct effect of polyamines on TORC1 and an indirect effect due to inhibition of a more distal target. To further explore this point, we tested if polyamines inhibited TORC1 through the EGOC. Overexpression of WT or signaling competent forms of Gtr1 and Gtr2 were not able to block the inhibitory effects of spermidine and spermine on TORC1 (Fig. 3.4.A), indicating that polyamines act downstream or in parallel of the Gtr's. The fact that spermidine and spermine suppress the residual TORC1 activity in cells lacking both Gtr1 and Gtr2 supports the second possibility (Fig. 3.4.A), but TORC1 *in vitro* kinase assay should be conduct to clarify this point.

We next investigated if Tor1 alleles conferring resistance to rapamycin and caffeine (Reinke et al., 2006) would also protect TORC1 from polyamines. Neither Tor1-1954V nor Tor1-1957V conferred spermine resistance to TORC1 (Fig. 3.4.B), indicating that polyamines inhibit TORC1 via a mechanism that is distinct from that of rapamycin or caffeine.

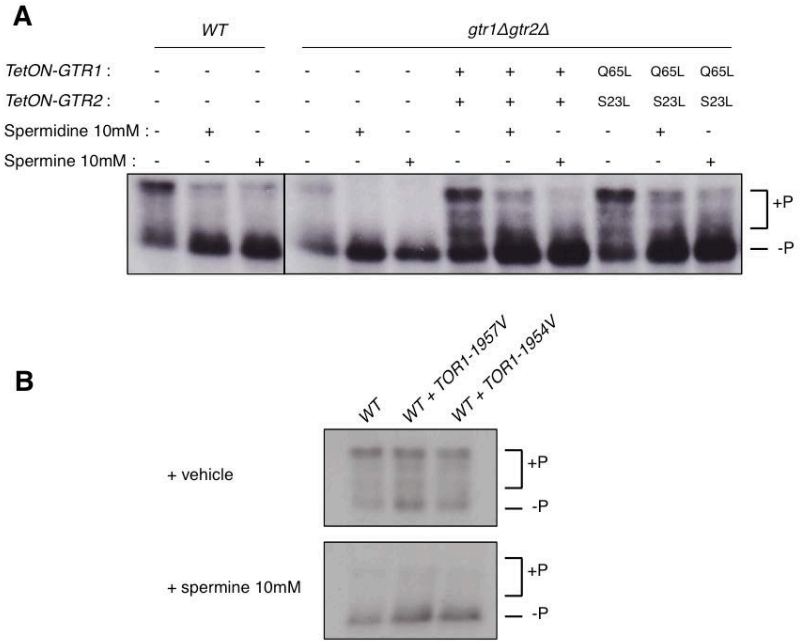

Fig.3.4. Polyamines inhibit TORC1 independently of EGOC. (**A, B**) Immunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 are shown (Urban et al., 2007). (A) Cells expressing the indicated construct swere treated with vehicle, spermidine, or spermine for 30 min. (B) Expression of rapamycin and caffeine resistant *TOR1* alleles did not confer resistance to spermidine.

To investigate whether perturbation of endogenous polyamine levels affected TORC1 activity we employed mutants that lacked components of the polyamine metabolic pathway (Fig. 3.2). Five of the six mutant strains tested grew similarly to a WT control strain on plates containing increasing concentrations of rapamycin and exhibited WT levels of TORC1 activity (Fig.3.5.A and B). However, these experiments were conducted in YPD medium; hence it is possible that mutants deficient for polyamine synthesis were able to import them from the environment. All mutant strains also exhibited typical levels of TORC1 activity in response to spermidine, spermine, rapamycin, or cycloheximide treatment and showed no defect in their ability to recover after rapamycin treatment (Fig.3.5.A and B). One notable exception was cells lacking the polyamine acetyl-transferase Paa1, which exhibited a slightly increased resistance to rapamycin compared to the WT (Fig. 3.5.A), which is in line with previous results (Xie et al., 2005). Interestingly, the *paa1Δ* mutant had been previously identified in screens for deletion strains exhibiting hypersensitivity to thialysine and to L-azetidine-2-carboxylic acid, which are toxic analogs of lysine and proline, respectively (Marie-Pierre Peli-Gulli and Floriane Jacquier, personal communication). Hypersensitivity to these compounds is also found in mutants with high TORC1 activity (Marie-Pierre Peli-Gulli, personal communication). Finally, Gtr1-TAP purification followed by mass-spectrometry identified Paa1 and the spermidine

synthase Spe3 as Gtr1 binding partners (Gregory Bonfils, personal communication), indicating that polyamine metabolism machinery could physically interact with TORC1 pathway components.

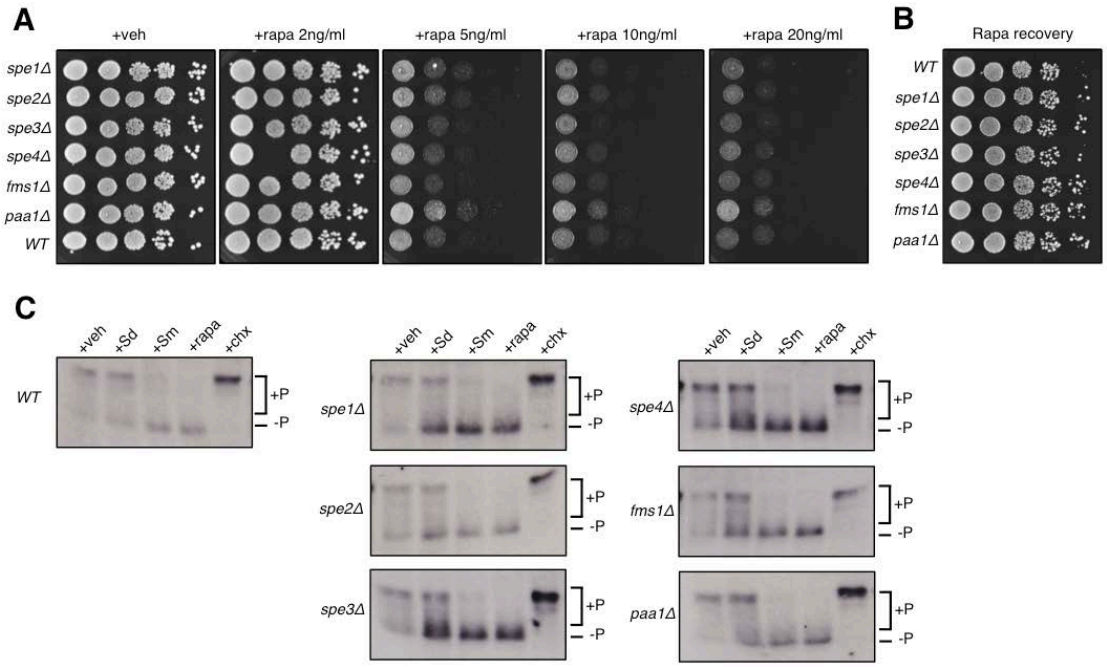

Fig.3.5. Characterization of mutants with impaired polyamine metabolism. (**A**) Wild-type and isogenic mutant cells were grown exponentially in synthetic medium complemented with 1 mM spermine and then spotted as 10-fold serial dilution on YPD plates containing the indicated amount of rapamycin. (**B**) Wild-type and isogenic mutant cells were grown as in (A), treated with 200 ng/ml of rapamycin during 6h, washed 2 times with YPD, and spotted in 10-fold serial dilutions on YPD plates. (**C**) TORC1 activity in the presence of different drugs was assessed in the same set of strains as in (A). Veh, Sd, Sm, Rapa and chx stand for vehicle, spermidine (10mM), spermine (10mM), rapamycin (200ng/ml), and cycloheximide (25μg/ml), respectively. Immunoblots detecting the extent of phosphorylation within the C-terminus of Sch9 are shown (Urban et al., 2007).

In conclusion, we have identified spermidine and spermine as TORC1 inhibitors that likely act downstream or in parallel of the EGO complex. *In vitro* kinase assays are now essential to determine if polyamines directly inhibit TORC1. Genetic screens for mutants resistant to spermidine or spermine could give valuable information on the mechanism by which polyamines inhibit TORC1, and could help to identify a novel TORC1 regulatory pathway that acts in parallel to the EGOC. Although several data implicate Paa1 in TORC1 regulation, precisely how Paa1 may acts upon and relays polyamine signals to TORC1 is unclear. Another pertinent question is why polyamines on one hand inhibit TORC1, increase lifespan and induce autophagy, and on the other hand are required for growth and present in excess in cancer.

3.3 Discussion

Recent developments in screening techniques and the increasing number of compounds available have allowed the discovery of promising molecules for use as therapeutic agents for TOR related diseases and as exceptional tools for fundamental research. Identification of CID 3528206 as a specific TORC1 inhibitor opens the way for further development of anticancer drugs and for identification of new TORC1 regulated processes. Polyamines may help to identify new TORC1 activators acting in parallel of the EGOC. Furthermore, understanding their mode of action and their role in cancer and ageing could help to identify new therapeutic targets in proliferative and age related diseases.

General Discussion

Part of the discussion has been published in Cell Cycle, Vol. 12 (issue 18).

Future directions for the study of SEACIT/SEACAT

SEACIT/GATOR1 and SEACAT/GATOR2 complexes form a new signaling branch that regulates Rag GTPases. Future studies should focus on deciphering how this branch is regulated and whether cues other than amino acids also impinge upon this branch. Here, I will discuss several areas that could be interesting to explore in order to investigate the regulation of SEACIT and SEACAT.

As discussed in the introduction, GAPs are typically regulated through their localization, expression or degradation rather than through their activity (Bigay et al., 2003; Canagarajah et al., 2004). In addition to its GAP domain, Iml1 is composed of three other conserved domains, namely DUF3608, DEP and the uncharacterized Domain 4 (Fig. 1.7.A). Interestingly, DEP (for Disheveled, EGL-10, Pleckstrin homology) domains are involved in the spatial regulation of numerous signaling molecules and interact at membranes with various partners such as phospholipids or membrane receptors (Consonni et al., 2014). It would be interesting to investigate if the Iml1 DEP domain could assume such a role and to determine potential binding partners. A version of Iml1 lacking the DUF3608 domain is unable to interact with Npr2 and Npr3 (Wu and Tu, 2011), thus it would be worth assessing the role of this domain with respect to the integrity and regulation of the SEACIT complex. Two phosphoserines (S680 and S737) have been identified in Iml1 in a large-scale study (Albuquerque et al., 2008). It would be interesting to determine whether the phosphorylation of these serines is regulated in response to amino acid levels and whether this would affect Iml1 localization, stability or interaction with its partners. If so, the next step would be to identify the kinase(s) responsible for the phosphorylation. Mck1, Kin2 and Fmp48 would be good candidates as they were found to interact with Iml1 in a large-scale mass spectrometric analysis (Breitkreutz et al., 2010).

Npr2 has been shown to be phosphorylated in a Yck1/Yck2-dependent manner (Spielewoy et al., 2010). Phosphorylated Npr2 is then targeted by the SCF^{Grr1} E3 ubiquitin ligase for degradation (Spielewoy et al., 2010). Strikingly, in the SPS amino acid sensing system, Yck1/Yck2 phosphorylates the Ssy5 prodomain upon amino acid stimulation (Abdel-Sater et al., 2011). This triggers the recognition of the Ssy5 prodomain by the SCF^{GRR1} ubiquitin ligase complex and its subsequent degradation (Abdel-Sater et al., 2011). As a consequence, the released Ssy5 catalytic domain processes the transcription factors Stp1 and Stp2, allowing their entry into the

nucleus. Therefore, it is possible that upon external amino acid stimulation, Ssy1 acts through Yck1/Yck2 on two branches: one would activate the transcriptional program of the SPS pathway and lead to the expression of amino acid permeases, while the other would activate TORC1 through the degradation of Npr2 and destabilization of SEACIT (Fig.4.1). This model is tempting, but further work is needed to determine if Npr2 is a direct target of Yck1/Yck2, if Ssy1 is involved in Npr2 phosphorylation and stability, and how SEACAT would impact on such a mechanism.

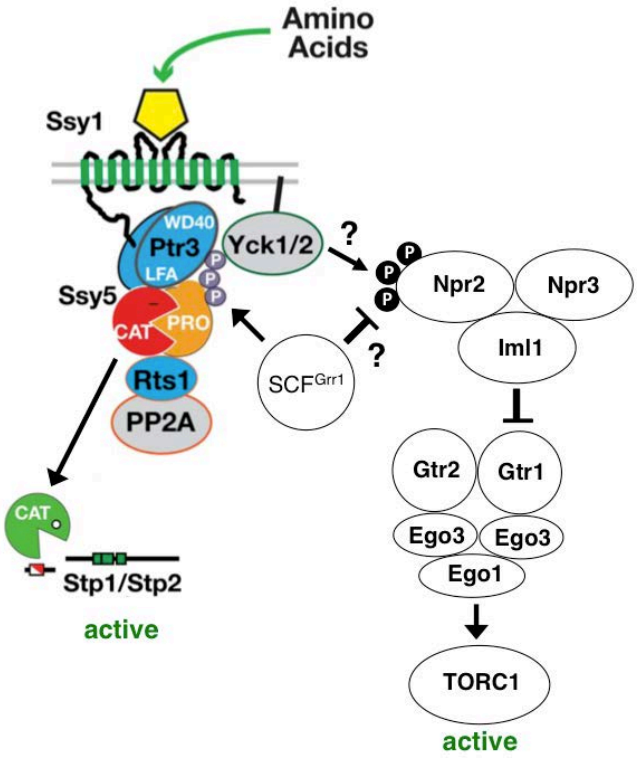

Fig.4.1 A model for the downregulation of SEACIT in response to external amino acids. Arrows represent activation events and bars indicate inhibition. Black balls containing a P denote phosphorylation. See text for more details. Adapted from (Omnus and Ljungdahl, 2013).

The group of Benjamin Tu recently reported another mechanism of regulation for Npr2 that involves methionine and the phosphatase PP2A (Sutter et al., 2013). Methionine promotes the synthesis of the methyl donor S-adenosylmethionine (SAM), which is required for methylation of the PP2A catalytic subunit. Methylated PP2A is then able to dephosphorylate Npr2 (Sutter et al., 2013). In the model of Tu's group, Npr2 phosphorylation promotes its interaction with Iml1 and has a positive effect on SEACIT (Wu and Tu, 2011). In contrast, Spielewoy *et al.* propose that Npr2 phosphorylation triggers its degradation and thus should destabilize SEACIT (Spielewoy et al., 2010). It is important to mention that Tu's group performed its experiments under very specific conditions: cells were grown in rich medium

containing lactate rather than dextrose as carbon source and were then switched to a minimal synthetic medium also containing lactate to induce waht they coin "non-nitrogen-starvation" (NNS) autophagy. It is possible that the different regulatory mechanisms involving Npr2 phosphorylation occur on distinct phosphosites, and depending on the environmental conditions, one mechanism is more prominent than the other. Mass spectrometric analysis in extracts from cells grown under different growth conditions would allow the identification of the exact sites targeted by the two different regulatory mechanisms.

How SEACAT inhibits SEACIT and how SEACAT is regulated are crucial outstanding questions that should be addressed. Curiously, both Sec13 and Seh1 not only function within the SEAC, but also within the nuclear pore complex (NPC) as part of the conserved heptameric Nup84 subcomplex that is essential for the overall architecture of the NPC and consequently the transport of mRNAs and macromolecules (e.g., pre-ribosomes) across the nuclear membrane (Hoelz et al., 2011). Moreover, Sec13 also associates with Sec31 into a heterotetramer, which forms the outer shell of coatmer complex II (COPII) coated vesicles of the secretory pathway that bud off from the endoplasmic reticulum (ER) (Gürkan et al., 2006; Zanetti et al., 2012). The occurrence of Sec13 and Seh1 in functionally different protein complexes suggests that their 3-dimensional structure, which is characterized, like those of all other SEACAT subunits, by the presence of WD-40 repeats that form β-propellers (Dokudovskaya and Rout, 2011; Dokudovskaya et al., 2011), renders them particularly well suited to serve as building and/or scaffolding blocks within larger protein complexes. Given these observations, it is tempting to speculate that Sec13/Seh1 serve to couple nuclear-to-cytoplasmic mRNA/protein transport or protein secretion to TORC1 control. For instance, compromised nuclear pore function or secretion may tie up or jam Sec13 and/or Seh1, thereby causing reduced SEAC assembly and consequently downregulation of TORC1. Interestingly, a genome-scale RNA interference screen by dsRNA reverse-transfection on living *Drosophila* cell microarrays identified nuclear pore components as TORC1 regulators (Lindquist et al., 2011). In a similar vein, alterations in the yeast secretory pathway have also been found to converge on TORC1 regulation. For instance, loss of the Golgi Ca^{2+}/Mn^{2+} ATPase Pmr1 strongly increased the secretion of (heterologous) proteins that transit through the secretory pathway and, based on genetic experiments, also caused TORC1 activation (e.g., *pmr1Δ* suppressed the rapamycin-sensitive phenotype of the *lst8-1* mutation) (Devasahayam et al., 2006; Rudolph et

al., 1989). Conversely, addition of the secretory pathway inhibitor tunicamycin and inactivation of the Rab escort protein Mrs6 both strongly inhibited TORC1-dependent phosphorylation of Sch9 (Lempiäinen et al., 2009; Loewith et al., 2002). In sum, these observations lend support to a model in which both NPC function and secretory pathway flux are part of an increasing number of physiological cues (including v-ATPase activity, leucyl-tRNA synthetase function, glutaminolysis-driven production of α-ketoglutarate, glucose and amino acid levels, vesicle trafficking, or actin polarization) (Binda et al., 2009; Bonfils et al., 2012; Durán et al., 2012; Efeyan et al., 2013; Flinn et al., 2010; Goranov et al., 2013; Han et al., 2012; Li et al., 2010; Zoncu et al., 2011), which may converge on Rag GTPase-mediated control of TORC1 (Fig.4.2). Future studies should therefore aim at deciphering whether any of these cues may fine-tune TORC1 by regulating the GTP loading status of Rag GTPases through the SEACIT/GATOR1 and/or SEACAT/GATOR2 complexes.

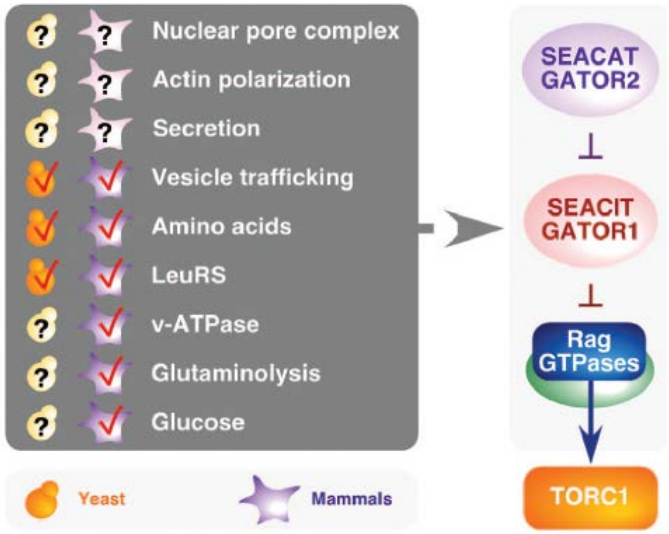

Fig.4.2. Physiological cues, which may regulate TORC1 through the Rag GTPase module. Red check marks indicate the existence of experimental data supporting (in yeast or mammalian cells) a model in which the respective cue impinges on Rag GTPase regulation (please see text for corresponding references). Currently speculative processes are denoted with a question mark.

Open questions concerning the regulation of EGOC/Ragulator

Several questions need to be answered in order to better understand the mechanism of TORC1 activation by EGOC/Ragulator. In yeast, TORC1 constitutively localizes to the vacuolar membrane in presence or absence of leucine (Binda et al., 2009) and the Rheb ortholog is not involved in yeast TORC1 activation. Thus, it remains to be

determined precisely how yeast TORC1 is activated. Is there a yet uncharacterized small GTPase playing a role similar to mammalian Rheb? Is the EGOC able to directly stimulate TORC1 kinase activity? A recent study in mouse embryonic fibroblasts reported that TORC1 activity not always correlates with its localization (Averous et al., 2014). Interestingly, in this study leucine controls TORC1 activity without affecting its lysosomal localization, whereas starvation or re-addition of all amino acids controls TORC1 localization and activity (Averous et al., 2014). Understanding how EGOC activates TORC1 in yeast without affecting its localization could help to decipher how leucine stimulates TORC1 in mammals.

Another mechanistic enigma is how Rag's recruit mTORC1 from the cytoplasm to the lysosome upon amino acid stimulation in mammals. So far Rag GTPases have been described to constitutively localize to the lysosomal periphery (Sancak et al., 2010). It would be interesting to investigate if a subset of Rag heterodimer could actually shuttle between the lysosomal surface and the cytoplasm. David Sabatini's group suggested a mechanism in which the GTP loading status of one Rag dictates the localization of the heterodimer whereas the conformation of the other Rag controls its interaction with TORC1 (Bar-Peled et al., 2012; Tsun et al., 2013). GDP-bound RagC has a primordial role in the interaction between the Rag heterodimer and mTORC1 (Tsun et al., 2013). Thus, we can imagine that the nucleotide-loading status of RagC/D would be responsible for its interaction with mTORC1 (*i.e.* interacting with mTORC1 when bound to GDP and releasing mTORC1 when bound to GTP) whilst the loading status of Rag A/B would regulate the localization of the heterodimer. Such a mechanism would explain why the RagC/D GAP, the FLCN-FNIP complex, is cytoplasmic (Tsun et al., 2013). GDP-bound RagB preferentially binds to Ragulator (Bar-Peled et al., 2012). Hence, we can speculate that GDP-bound RagA/B would promote the localization of the heterodimer at the lysosome, whereas a heterodimer containing GTP-bound RagA/B would localize in the cytosol. However, this would not explain why GTP-locked RagA/B has a dominant effect on mTORC1 activation and renders mTORC1 insensitive to amino acids deprivation (Sancak et al., 2008). On the other hand, in yeast, a nucleotide-free form of Gtr1 interacts less strongly with the EGOC than its WT or GTP-locked counterpart (Binda et al., 2009). A model in which a heterodimer containing GTP-bound RagA/B localizes at the lysosome and one containing GDP-bound RagA/B localizes in the cytosol would imply that the RagA/B GEF should be cytoplasmic, which is not the case of Ragulator (Sancak et al., 2010).

A recent study showed that upon amino acid withdrawal, Rheb GTP needs to switch to the GDP bound form in order to fully release mTORC1 from the lysosome. To accelerate the Rheb hydrolysis reaction, inactive Rag GTPase heterodimers recruit the TSC complex to the lysosome (Demetriades et al., 2014). In other words, the TSC complex is also part of the amino acid signaling machinery to mTORC1 and Rheb is also involved in mTORC1 localization. This finding showed that the mechanisms regulating mTORC1 activation/inactivation are more complex than the initially postulated model where Rag GTPases regulate mTORC1 localization and Rheb mTORC1 activity. It also blurs the line separating the mTORC1 amino acid sensing branch from the growth factor sensing branch. In this optic it would be worth to investigate if other cross talk is taking place between this two branches, especially if compounds of the growth factor branch could regulate localization of the RagA/B GAP complex GATOR1 or the RagC/D GAP complex FLCN-FNIP1. It would also be interesting to test if Gtr1/Gtr2 in yeast are required for TORC1 inactivation. Such a role would explain why expression of a GDP-bound allele of Gtr1 is dominant negative with respect to TORC1 activity, irrespectively of the nucleotide binding status of Gtr2 (Binda et al., 2009).

In conclusion, the heterodimerization of two GTPases that need to be asymmetrically loaded to be active is a unique mechanism. The fact that it has been fixed very early in eukaryotic evolution demonstrates its efficiency. However, this mechanism has not revealed all of its secrets and still promises many exciting discoveries.

Implication of mTORC1 amino acid sensing branch in human diseases

As mTORC1 is a central signaling hub, misregulation of its activity can lead to dramatic consequences. Numerous mutations in components impinging on the Rheb-TSC module, as well as mutations in Rheb and the TSC complex themselves, have been linked to neurological diseases, cancer, diabetes and inflammation (Costa-Mattioli and Monteggia, 2013; Dazert and Hall, 2011; Inoki et al., 2005; Takei and Nawa, 2014; Zoncu et al., 2010). Accumulating evidence show that the amino acid sensing branch of mTORC1 plays an important physiological role and its misregulation is linked to several pathologies.

First of all, Rag GTPases regulate cell and organ size in Drosophila (Kim et al., 2008; Resnik-Docampo and de Celis, 2011). In mouse, absence of RagA, but not RagB, results in embryonic death after 10.5 days of development and ablation of RagA in

adult mice is lethal (Efeyan et al., 2014). Mice expressing a GTP-locked allele of RagA develop normally but die in the first day after their birth because they fail to induce autophagy (Efeyan et al., 2012). Mice lacking the Ragulator components p14 or p18 die around embryonic day 7-8 (Nada et al., 2009; Teis et al., 2006). In humans, a mutation that reduces p14 expression causes a primary immunodeficiency syndrome and severe growth defect (Bohn et al., 2007). Cells isolated from patients carrying this mutation exhibit low mTORC1 activity (Sancak et al., 2010). Loss-of-function mutations in the RagC/D GAP subunit FLCN are responsible for a familial syndrome called Birt-Hogg-Dubé (BHD), which causes non-cancerous tumors of the hair follicules (fibrofolliculomas), kidney and lung (Nickerson et al., 2002; Tsun et al., 2013). The symptoms of BHD syndrome are similar to those of tuberous sclerosis, a disease caused by mutations in the TSC complex (Huang and Manning, 2008). Both the TSC complex and Folliculin act as tumor suppressors, but intriguingly they have opposite effects on mTORC1. How can a positive regulator of mTORC1 such as FLCN be a tumor suppressor? A possible explanation is that inactivation of mTORC1 triggers hyperactivation of other pathways such as the PI3K/Akt pathway and/or the mTORC2 signaling branch.

As previously discussed, GATOR1 components DEPDC5, NPRL2 and NPRL3 are frequently lost or mutated in cancer (Bar-Peled et al., 2013). Several studies now link DEPDC5 to epilepsy. Different mutations in DEPDC5 have been identified in families harboring genetic focal epilepsy (Dibbens et al., 2013; Ishida et al., 2013; Lal et al., 2014; Martin et al., 2013; Picard et al., 2014; Scheffer et al., 2014). Most of the identified mutations are nonsense or frameshift, and for two of them it has been shown that the truncated transcripts are degraded by the Non-sense-mediated mRNA Decay (NMD) pathway (Picard et al., 2014). Therefore, it appears that DEPDC5-related epilepsy is caused by haploinsufficiency (Picard et al., 2014). However, exome sequencing allowed the identification of a 55 amino acid in-frame deletion in a patient (Picard et al., 2014). Interestingly, the missing region (R785 to G839) is located in the domain corresponding to the Iml1 GAP-domain, and lacks the conserved arginine-containing domain that we identified as essential for Iml1 GAP activity (Fig.1.7.A). TORC1 hyperactivation in the brain appears to play a critical role in epilepsy as patients with TSC-complex mutations are also subject to seizures (Russo et al., 2012). Hence, the life of patients having TSC or DEPDC5-related epilepsy could be improved by treatment with the clinically approved rapamycin. Finally, a screen in yeast for molecules that inhibit the growth of *iml1Δ* cells, but not

the growth of cells expressing a GTP-locked allele of Gtr1, could allow the identification of specific RagA/B/Gtr1 inhibitors. Such molecules could be more specific than rapamycin or other TORC1 inhibitors for the treatment of patients with DEPDC5-related epilepsy or with tumors containing mutations in GATOR1.

Materials and Methods

Growth conditions

Unless stated otherwise, prototrophic strains were pre-grown overnight in synthetic dropout medium (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mix and 2% glucose). Before each experiment, cells were diluted to an OD_{600} of 0.2 and further grown at 30°C 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 medium with leucine (0.37 mg ml^{-1}), filtered, washed twice, and resuspended in same medium devoid of leucine. For galactose induction, precultures were grown in synthetic medium (SRaf; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mix, 2% raffinose and 0.1% sucrose). Cells were then diluted to an OD_{600} of 0.2 and further grown in SRaf supplemented with 2% galactose until they reached an OD_{600} of 0.8. Expression of genes under the control of the Tet_{ON} promoter was induced by adding 5μg ml^{-1} doxycycline to specified medium. *S. cerevisiae* strains and plasmids used in this study are listed in the tables in the next section.

Chemicals

Rapamycin was purchased from LC laboratories. Cycloheximide was purchased from Fluka. GTP, GDP, GTPγS, NaF, $AlCl_3$, spermine, spermidine, N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), 2-Nitro-5-thiocyanato-benzoic acid (NTCB) and 1,3-dihydro-1-hydroxy-2,1-bentoxaborole (DHBB) were purchased from Sigma-Aldrich.

TORC1 activity assays

TORC1 activity was quantified by assessing the phosphorylation of the C-terminal part of HA-tagged $Sch9^{T570}$ which contains at least five *bona fide* TORC1 phosphorylation sites, as described previously (Binda et al., 2009; Urban et al., 2007). Briefly, following chemical cleavage with NTCB, extracts were separated by 7.5% SDS-PAGE and membranes were probed with anti-HA antibodies (12CA5) and anti-mouse IgG antibodies coupled to HRP (Biorad). Alternatively (in Fig. 1B), TORC1 activity was estimated as the ratio between the phosphorylation on Thr^{737} of full length Sch9-HA_5 compared to the total abundance of Sch9-HA_5 (using phosphospecific anti-$pThr^{737}$-Sch9 and 12CA5 antibodies, respectively) as previously described (Takahara and Maeda, 2012).

Co-immunoprecipitation

Yeast cells expressing the indicated fusion proteins were harvested by filtration. Filters were immediately snap frozen in liquid nitrogen and stored at -80°C. Cells were resupended in lysis buffer (50 mM Hepes/KOH pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.2% NP40 (for myc-Iml1) or 0.5% NP40 (for Kog1-HA), protease and phosphatase inhibitor cocktails (Roche)) and lysed with glass beads using the Precellys cell disruptor. Lysates were diluted in the same lysis buffer (for myc-Iml1) or a buffer devoid of NP40 (for Kog1-HA), and clarified by two successive centrifugations for 10 min at 13 000 rpm. For input samples, aliquots of cleared lysates were concentrated by precipitation with ice-cold acetone, resuspended in 6x concentrated loading buffer and denatured 10 min at 95°C. For co-immunoprecipitations, cleared lysates were incubated for two hours at 4°C with prewashed IgG Sepharose beads (GE Healthcare). Following three washes with wash buffer (same as lysis buffer for myc-Iml1, or containing 450 mM NaCl for Kog1-HA), beads were resupended in 6x concentrated loading buffer and denatured for 10 min at 95°C. Inputs (25 μg) and pulldown samples (2000 μg for myc-Iml1, 500 μg for Kog1-HA and 125 μg or 67.5 μg for Gtr1-TAP) were analyzed by SDS-PAGE-immunoblot using anti-myc (9E10 ; Santa Cruz), anti-HA (HA.11 ; Covance) and anti-TAP (Open Biosystems) antibodies together with light-chain specific anti-mouse or anti-rabbit HRP-conjugated antibodies (Jackson Immuno Research).

Protein purification

GST-Gtr1, GST-Gtr2, GST-Gtr2 (S23L or Q66L), Gtr1- His_6, GST-Cdc42, and GST-$Iml1^{GAP}$ were produced in the *E. coli* Rosetta^{TM} strain after induction with 0.5 mM IPTG during 5 hours at 18°C (GST-Gtr1, GST-Gtr2, and GST-Gtr2 (S23L or Q66L) plus Gtr1- His_6), 30°C (GST-$Iml1^{GAP}$) or 37°C (GST-Cdc42). Cells were collected by centrifugation and lysed with a microfluidizer. Protein fusions were purified by using Glutathione-Sepharose beads (GE Healthcare), which were washed with buffer A (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.1% NP40, and 0.1 mM GDP) for GST-Gtr1, GST-Gtr2, GST-Gtr2 (S23L or Q66L) plus Gtr1- His_6, and GST-Cdc42, or buffer B (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 1% Triton X-100, and 0.1% Tween) for GST-$Iml1^{GAP}$. Proteins were eluted with Buffer A plus 10 mM reduced glutathione (without GDP in the case of GST-$Iml1^{GAP}$). Glycerol was added to a final concentration of

20%. Proteins were snap frozen in liquid nitrogen and stored at -80°C.

Codon optimized version of *His_6-EGO1*, *EGO2*, *EGO3* and *EGO4* were co-expressed in *E. coli* Rosetta^{TM} strain after induction with 0.5 mM IPTG during 3 hours at 30°C. Cells were collected by centrifugation, resuspended in Buffer C (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 20 mM imidazole) and lysed with a microfluidizer. The lysate was passed through a 1ml HisTrap FF crude column from GE Healthcare, washed with Buffer C and eluted with Buffer C containing 500mM imidazole. The sample was further purified through a 16/60 Superdex 200 column from GE Healthcare equilibrated with Buffer D (20 mM HEPES, 150 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, pH 7.5). The pufified EGO complex was concentrated by centrifugation with a vivaspin column (10'000 MWCO, Sartorius), glycerol was added to a final concentration of 20%, and proteins were snap frozen in liquid nitrogen and stored at -80°C.

For the purification of full length Iml1 and Iml1^{R943A}, yeast *iml1Δ gtr1Δ* double mutant cells expressing Iml1- or Iml1^{R943A}-His_6-TEV-ProtA were grown in synthetic dropout medium overnight, diluted to an OD_{600} of 0.2 in YPD and further grown to an OD_{600} of 2.0. Cells were then collected by centrifugation and lysed with a planetary micro mill (Pulverisette). Protein fusions were purified using IgG Sepharose beads (GE Healthcare), which were washed with buffer E (20 mM HEPES pH 7.4, 300 mM NaCl, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% tween, 1% Triton X-100, 1 mM DTT) (Dokudovskaya et al., 2011). Following overnight TEV cleavage, a second purification step was performed using Ni-NTA Agarose beads (Qiagen), which were washed with Buffer C plus 10 mM imidazole. Proteins were eluted with Buffer F (20 mM HEPES pH 7.4, 75 mM NaCl, 110 mM KOAc, 2 mM $MgCl_2$, and 300 mM imidazole). Glycerol was added to a final concentration of 20%. Proteins were snap frozen in liquid nitrogen and stored at -80°C.

In vitro Gtr1-Iml1 binding assays

Purified GST-Gtr1 or GST alone (100 nM final) was incubated for 30 min at room temperature in loading buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, and 1 mM DTT) in the presence of either GTPγS (100 µM final), GTP (1 mM final), GDP (1 mM final), or GDP + AlF_x (1 mM GDP, 2 mM $AlCl_3$, and 20 mM NaF final concentration). Subsequently, purified Iml1-His_6 (100 nM) and $MgCl_2$ (10 mM) were added and the mix was incubated for 1 hour at 4°C. Pulldown experiments were performed using Glutathione-Sepharose beads (Qiagen), which were washed with loading buffer

containing 10 mM $MgCl_2$ (or 10 mM $MgCl_2$, 2 mM $AlCl_3$ and 20 mM NaF; GDP plus AlF_x). Finally, beads were resuspended in 2x Laemmli buffer, boiled and the supernatants were used for SDS-immunoblot analyses.

GTP hydrolysis assays

GAP assays were performed essentially as previously described (Anderson and Chamberlain, 2005; Chamberlain et al., 2004). Briefly, GTPases (100 nM) were incubated for 30 min at room temperature in loading buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, and 1 mM DTT) in presence of 40 nM [a-^{32}P]-GTP (Harman Analytic, 3000 Ci/mmol). Full length Iml1-His_6/Iml1^{R943A}-His6, or GST-Iml1^{GAP}/GST-Iml1$^{GAP\text{-}R943A}$ were then added to the mixtures (at the indicated concentrations), together with 10 mM $MgCl_2$ to initialize the reactions. After 20 minutes of incubation at room temperature, reactions were stopped by addition of elution buffer (1% SDS, 25 mM EDTA, 5 mM GDP, and 5 mM GTP) and heating for 2 minutes at 65°C. [a -^{32}P]-GTP and [a-^{32}P]-GDP were separated by TLC on PEI Cellulose F plates (Merck) with buffer containing 1 M acetic acid and 0.8 M LiCl. Results were visualized using a phosphorimager and quantified with ImageQuant. Single turnover GAP assays were performed with Iml1-His_6 (200 nM), Iml1^{R943A}-His_6 (200 nM), GST-Iml1^{GAP} (1 μM), or GST-Iml1$^{GAP\text{-}R943A}$ (1 μM) as described above except that 1.7 mM unlabeled GTP was added simultaneously with $MgCl_2$.

Microscopic analyses

Mid-log phase cells cultured in specified synthetic dropout medium 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 containing the filter sets U-MWIBA, U-MWIG and U-MNUA2 (Olympus). Images were acquired with a F-view2 camera (Olympus). CellM software (Olympus) was used to control the microscope and the camera. 7 to 10 z-section separated from 0.5 μm were acquired and then projected to two-dimentional images and analyzed with CellM software.

Split-ubiquitin yeast two-hybrid assay

The membrane-based yeast two-hybrid system provided by Dualsystems Biotech was used following the manufacturer instructions to assay protein-protein interactions. Briefly, the NMY51 strain containing the LexAop-HIS3, LexAop-ADE2

and LexAop-LacU reporter genes was co-transformed with a bait plasmid (pCAB-WT) expressing a CUB-LexA-DBD fusion protein from the CYC1 promoter and a prey plasmid (pPR3-N) expression a NUBG-HA fusion protein from the CYC1 promoter. Cells were grown to mid-log phase in synthetic medium without leucine and tryptophan. Classical β-Galactosidase assays using ONPG were performed (Stern et al., 1984) and interactions were calculated in Miller Units.

Table 1. Strains used in the chapter I

Strain	Genotype	Source	Figure
YL515	[BY4741/2]*MATα*; *his3Δ1, leu2Δ0, ura3Δ0*	Binda, 2009	1.1A-D; 1.2A-C; 1.3A; 1.4A-C,E; 1.7A-D; 1.9; 1.10A
YL516	[BY4741/2]*MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0*	Binda, 2009	1.4D
NP04-4C	[YL515] *MATα*; *iml1Δ::kanMX4*	Panchaud, 2013	1.1A-D; 1.2A-C; 1.3A; 1.4A, B, E ; 1.7D, E; 1.10A; 1.11
NP15-1B	[YL515] *MATα*; *npr2Δ::kanMX4*	Panchaud, 2013	1.1A-D; 1.3A; 1.4A, E; 1.11
NP23-4D	[YL515] *MATα*; *npr3Δ::kanMX4*	Panchaud, 2013	1.1A-D; 1.3A; 1.4A, E; 1.11
NP12-1D	[YL515] *MATα*; *sea2Δ::kanMX4*	Panchaud, 2013	1.1A, C, D
NP13-7A	[YL515] *MATα*; *sea3Δ::kanMX4*	Panchaud, 2013	1.1A, C, D
NP14-3C	[YL515] *MATα*; *sea4Δ::kanMX4*	Panchaud, 2013	1.1A, C, D
NP21-1B	[YL515] *MATα*; *npr2Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.2A; 1.4E
MP282-9A	[YL515] *MATα*; *iml1Δ::kanMX4,npr2Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.1A, B; 1.2A; 1.4E
NP33-2B	[YL515] *MATα*; *sea2Δ::kanMX4, sea3Δ::kanMX4, sea4Δ::kanMX4*	Panchaud, 2013	1.1A, B; 1.2A
NP35-9D	[YL515] *MATα*; *iml1Δ::kanMX4, sea2Δ::kanMX4, sea3Δ::kanMX4, sea4Δ::kanMX4*	Panchaud, 2013	1.2A; 1.3C; 1.11
NP37-7A	[YL516] *MAT***a**; *npr2Δ::kanMX4, sea2Δ::kanMX4, sea3Δ::kanMX4, sea4Δ::kanMX4*	Panchaud, 2013	1.2A; 1.11
NP38-6B	[YL515] *MATα*; *npr3Δ::kanMX4, sea2Δ::kanMX4, sea3Δ::kanMX4, sea4Δ::kanMX4*	Panchaud, 2013	1.2A; 1.11
KT1961	*MAT**a***; *his3, leu2, ura3-52, trp1*	Pedruzzi, 2003	1.3C
MP240-1C	[KT1961] *MAT***a**; *iml1Δ::kanMX4*	Panchaud, 2013	1.3B; 1.5C
MP294-8B	[KT1961] *MAT***a**; *iml1Δ::kanMX4, npr2Δ::kanMX4*	Panchaud, 2013	1.3B
MP295-6B	[KT1961] *MAT***a**; *iml1Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.3B
MP296-5B	[KT1961] *MAT***a**; *iml1Δ::kanMX4, npr2Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.3B
MP285-1A	[KT1961] *MAT***a**; *npr2Δ::kanMX4*	Panchaud, 2013	1.3B; 1.5C
MP287-5B	[KT1961] *MAT***a**; *npr2Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.3B
MP286-1B	[KT1961] *MAT***a**; *npr3Δ::kanMX4*	Panchaud, 2013	1.3B; 1.5C
MP290-2D	[KT1961] *MAT***a**; *ego1Δ::kanMX4, ego3Δ::kanMX4, gtr1Δ::natMX4, gtr2Δ::natMX4*	Panchaud, 2013	1.3B
MB27	[YL515] *MATα*; *gtr1Δ::HIS3*	Binda, 2009	1.4A
MB28	[YL515] *MATα*; *gtr2Δ::HIS3*	Binda, 2009	1.4A
MB34	[YL515] *MATα*; *tco89Δ::HIS3*	Binda, 2009	1.4A
MP202-1C	[YL515] *MATα*; *gtr1Δ::HIS3, iml1Δ::kanMX4*	Panchaud, 2013	1.4A; 1.5G; 1.6A, B; 1.7C, F; 1.8C, D; 1.10B
MP206-5B	[YL515] *MATα*; *gtr2Δ::HIS3, iml1Δ::kanMX4*	Panchaud, 2013	1.4A

Table 1. Strains used in the chapter I – continued

Strain	Genotype	Source	Figure
MP203-5A	[YL515] *MATα*; *tco89Δ::HIS3, iml1Δ::kanMX4*	Panchaud, 2013	1.4A
NP17-4H	[YL515] *MATα*; *gtr1Δ::kanMX4, npr2Δ::kanMX4*	Panchaud, 2013	1.4A
NP19-2B	[YL515] *MATα*; *gtr1Δ::HIS3, npr3Δ::kanMX4*	Panchaud, 2013	1.4A
NP03-4B	[YL516] *MAT***a**; *iml1Δ::kanMX4*	Panchaud, 2013	1.4D
MB32	[YL516] *MAT***a**; *gtr1Δ::kanMX4*	Binda, 2009	1.4D, E
MP242-2D	[YL516] *MAT***a**; *MYC-IML1, IGO1-TAP::KanMX4*	Panchaud, 2013	1.5A
MP241-2C	[YL516] *MAT***a**; *MYC-IML1, gtr1Δ::HIS3*	Panchaud, 2013	1.5A, B, E
MP306-16A	[YL516] *MAT***a**; *MYC-IML1, gtr1Δ::HIS3, npr2Δ::kanMX4, npr3Δ::kanMX4*	Panchaud, 2013	1.5B
MJA351-2A	[KT1961] *MAT***a**; *iml1::IML1-VN-TRP1, gtr1::GTR1-VC-HIS3*	Panchaud, 2013	1.5D
MP256-5B	[YL515] *MATα*; *kog1::KOG1-HA-KanMX4, gtr1Δ::HIS3*	Panchaud, 2013	1.5F
MP308-7A	[YL515] *MATα*; *seh1Δ::kanMX4*	Panchaud, 2013	1.2B
MP308-8B	[YL515] *MATα*; *seh1Δ::kanMX4, iml1Δ::kanMX4*	Panchaud, 2013	1.2B
MP309-5D	[YL515] *MATα*; *sec13^{ts}-kanMX4*	Panchaud, 2013	1.2B
MP309-9A	[YL515] *MATα*; *sec13^{ts}-kanMX4, iml1Δ::kanMX4*	Panchaud, 2013	1.2B
MP06-8B	[YL515] *MATα*; *gtr1Δ::kanMX4, gtr2Δ::kanMX4*	Binda, 2009	1.10A

Table 2. Plasmids used in the chapter I

Plasmid	Description	Source	Figure
pRS413	CEN, *HIS3*	Brachman, 1998	1.1A, B; 1.3; 1.4A; 1.5A, B, G; 1.6; 1.7C, F; 1.8C, D; 1.10A; 1.11
pRS414	CEN, *TRP1*	Brachman, 1998	1.3A, B; 1.5C
pRS415	CEN, *LEU2*	Brachman, 1998	1.1A, C, D; 1.2A-C; 1.3B, C; 1.4A-C; 1.5A, B, D; 1.7A, D, E; 1.9
pRS416	CEN, *URA3*	Brachman, 1998	1.1; 1.2; 1.3A; 1.4B-E; 1.5A, D; 1.7D, E; 1.9; 1.10A
pJU1064	[pRS413] $SCH9^{T570A}$-HA_5	Urban, 2007	1.1C, D; 1.2; 1.4B-E; 1.9; 1.7D, E; 1.10A
pJU1058	[pRS415] $SCH9^{T570A}$-HA_5	Urban, 2007	1.3A; 1.10A
pJU1030	[pRS416] $SCH9^{T570A}$-HA_5	Urban, 2007	1.4A; 1.7A
BG1805	2µ, *URA3, GAL1p*	Open Biosystems	
pNP1946	[BG1805] *IML1- HIS_6*	Panchaud, 2013	1.3A; 1.4C; 1.9
pNP2094	[BG1805] *NPR2- HIS_6*	Panchaud, 2013	1.3A
pNP2095	[BG1805] *NPR3- HIS_6*	Panchaud, 2013	1.3A
pNP2106	[BG1805] *NPR3- HIS_6, ura3::HIS3*	Panchaud, 2013	1.3A
YCplac111	CEN, *LEU2*	Gietz, 1998	
pDK2698	[YCplac111] *ADH1p-SYO1-$(GA)_5$-3xEGFP*	Kressler, 2012	
pDK2712	[YCplac111] *ADH1p-3xEGFP-$(GA)_5$-RIX7*	D. Kressler	
pMP2118	[YCplac111] *IML1p-IML1-$(GA)_5$-3xEGFP, leu2::URA3*	Panchaud, 2013	1.3B, C; 1.5C; 1.11
pNP2350	[pRS416] *IML1p-IML1-R943A-$(GA)_5$-3xEGFP*	Panchaud, 2013	1.4E
pFLJ2286	[pRS416] *NPR2p-NPR2-$(GA)_5$-3xEGFP*	Panchaud, 2013	1.3B; 1.5C; 1.11
pFLJ2293	[pRS416] *NPR3p-3xEGFP-$(GA)_5$-NPR3*	Panchaud, 2013	1.3B; 1.5C; 1.11
pMP2149	[pRS416] *CYC1p-HHF2-Tdimer2(12), ura3::HIS3*	Panchaud, 2013	1.3C
YCplac33	CEN, *URA3*	Gietz, 1988	
pMB1393	[YCplac33] *Tet_{ON}-GTR1 (GTR1-WT)*	Binda, 2009	1.4B
pMB1394	[YCplac33] *Tet_{ON}-$GTR1^{Q65L}$ (GTR1-GTP)*	Binda, 2009	1.4B, D, E; 1.10A
pMB1395	[YCplac33] *Tet_{ON}-$GTR1^{S20L}$ (GTR1-GDP)*	Binda, 2009	1.4B
pPM1621	[YCplac111] *Tet_{ON}-GTR2 (GTR2-WT)*	Panchaud, 2013	1.4B
pPM1622	[YCplac111] *Tet_{ON}-$GTR2^{Q66L}$ (GTR2-GTP)*	Panchaud, 2013	1.4B, C
pPM1623	[YCplac111] *Tet_{ON}-$GTR2^{S23L}$ (GTR2-GDP)*	Bonfils, 2012	1.4B, C; 1.10A
pPM1397	[YCplac111] *Tet_{ON}-$GTR1^{Q65L}$ (GTR1-GTP)*	Panchaud, 2013	1.4C
pPM1398	[YCplac111] *Tet_{ON}-$GTR1^{S20L}$ (GTR1-GDP)*	Panchaud, 2013	1.4C
pMB1344	[YCplac33] *GTR1p-GTR1-TAP*	Binda, 2009	1.5A, B, E, F
pMB1372	[YCplac33] *GTR1p-$GTR1^{Q65L}$ –TAP*	Binda, 2009	1.5E, F
pRS425	2µ, *LEU2*	Christianson, 1992	
pFLJ2251	[pRS425] *GAL1p-IML1*	Panchaud, 2013	1.5F
pJU650	[pRS416] *GTR1p-GTR1*	R. Loewith	1.5B, F
pDK2261	[YCplac111] *NSA1p-NSA1- HIS_6-TEV-ProtA*	D. Kressler	

Table 2. Plasmids used in the chapter I - continued

Plasmid	Description	Source	Figure
pNP2055	[YCplac111] *ADH1p-IML1- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.5F; 1.6; 1.7C, E, F; 1.8C, D; 1.10B
pNP2125	[YCplac111] *ADH1p-IML1-R273A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2126	[YCplac111] *ADH1p-IML1-Q284A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2127	[YCplac111] *ADH1p-IML1-R519A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2128	[YCplac111] *ADH1p-IML1-R943A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7C-F
pNP2129	[YCplac111] *ADH1p-IML1-R1009A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2130	[YCplac111] *ADH1p-IML1-R1059A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2131	[YCplac111] *ADH1p-IML1-R1077A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2132	[YCplac111] *ADH1p-IML1-R1109A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2133	[YCplac111] *ADH1p-IML1-R1400A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2134	[YCplac111] *ADH1p-IML1-R1499A- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.7D
pNP2285	[pRS416] *ADH1p-IML1- HIS$_6$-TEV-ProtA*	Panchaud, 2013	1.8C, D
pFLJ1931	[pET-24d] *GST-TEV*	Panchaud, 2013	1.5G; 1.7F
pNP2035	[pET-24d] *GST-TEV-GTR1*	Panchaud, 2013	1.5G; 1.6A, B; 1.7B, C, F; 1.8A, C, D; 1.10B
pNP2038	[pET-24d] *GST-TEV-GTR2*	Panchaud, 2013	1.6B
pNP2159	[pET-24d] *GST-TEV-CP1*	This study	1.10B
pNP2160	[pET-24d] *GST-TEV-CP1^{S414F}*	This study	1.10B
pMP2101	[pGEX-4T] *GST-CDC42*	Panchaud, 2013	1.6B
pNP2137	[pET-24d] *GST-TEV-IML1^{GAP}(877-1178)*	Panchaud, 2013	1.7B, C
pNP2142	[pET-24d] *GST-TEV-IML1^{GAP}(877-1178)-R943A*	Panchaud, 2013	1.7B, C
pJU1013	[pET-28a] *GTR1-LE-HIS$_6$*	R. Loewith	1.8C, D
pJU1047	[pGEX-6P] *GST-TEV-GTR2^{S23L}-HIS$_6$*	R. Loewith	1.8C, D
pJU1048	[pGEX-6P] *GST-TEV-GTR2^{Q65L}-HIS$_6$*	R. Loewith	1.8C, D
pRS426	2µ, *URA3*	Christianson 1992	
pFLJ1981	[pRS426] *GAL1p-IML1(1-1548)*	Panchaud, 2013	1.7A
pFLJ1982	[pRS426] *GAL1p-IML1(1-1274)*	Panchaud, 2013	1.7A
pFLJ1983	[pRS426] *GAL1p-IML1(1-1184)*	Panchaud, 2013	1.7A
pFLJ1984	[pRS426] *GAL1p-IML1(585-1184)*	Panchaud, 2013	1.7A
pFLJ1985	[pRS426] *GAL1p-IML1(113-585)*	Panchaud, 2013	1.7A
pFLJ1986	[pRS426] *GAL1p-IML1(1184-1548)*	Panchaud, 2013	1.7A
pFLJ1987	[pRS426] *GAL1p-IML1(1284-1548)*	Panchaud, 2013	1.7A
YCplac195	2µ, *URA3*	Gietz 1988	
pNP2208	[YCplac195] *GAL1p-DEPDC5- HIS$_6$*	Panchaud, 2013	1.9

Table 3. Strains used in the chapter II

Strain	Genotype	Source	Figure
YL515	[BY4741/2]*MATα*; *his3Δ1, leu2Δ0, ura3Δ0*	Binda, 2009	2.2
MB27	[YL515] *MATα*; *gtr1Δ::HIS3*	Binda, 2009	2.2A, B; 2.3
MB28	[YL515] *MATα*; *gtr2Δ::HIS3*	Binda, 2009	2.2A, B; 2.3
NP52-2A	[YL515] *MATα*; *ego2Δ::KanMX*	This study	2.2A, B; 2.4A
NP44	[YL515] *MATα*; *ego4Δ::KanMX*	This study	2.2A, B; 2.4A
MP7-2B	[YL515] *MATα*; *vam6Δ::KanMX*	This study	2.2A, B
NP51-3C	[YL515] *MATα*; *ego2Δ::KanMX, gtr1Δ::KanMX*	This study	2.2A, B; 2.3
NP54-4D	[YL515] *MATα*; *ego2Δ::KanMX, gtr2Δ::KanMX*	This study	2.2A, B; 2.3
NP56-8A	[YL515] *MATα*; *ego2Δ::KanMX, vam6Δ::KanMX*	This study	2.2A, B
NP48-5C	[YL515] *MATα*; *ego4Δ::KanMX, gtr1Δ::KanMX*	This study	2.2A, B; 2.3
NP60-10D	[YL515] *MATα*; *ego4Δ::KanMX, gtr2Δ::KanMX*	This study	2.2A, B; 2.3
NP50-4C	[YL515] *MATα*; *ego4Δ::KanMX, vam6Δ::KanMX*	This study	2.2A, B
NMY51	*MAT**a**; his3Δ200, trp1-901, leu2-3,112, ade2, LYS2::(lexAop)4-HIS3, ura3::(lexAop)8- lacZ, ade2::(lexAop)8-ADE2, GAL4*	Dual-System Biotech AG	2.4B

Table 4. Plasmids used in the chapter II

Plasmid	Description	Source	Figure
pRS413	CEN, *HIS3*	Brachman, 1998	2.2A, B; 2.3
pRS415	CEN, *LEU2*	Brachman, 1998	2.2C
pRS416	CEN, *URA3*	Brachman, 1998	2.2; 2.3
pJU1064	[pRS413] $SCH9^{T570A}$-HA_5	Urban, 2007	2.2C
pJU1058	[pRS415] $SCH9^{T570A}$-HA_5	Urban, 2007	2.2A, B
YCplac111	CEN, *LEU2*	Gietz, 1998	
pNP2529	[Ycplac111] *ADH1p-EGO2*	This study	2.2C
pNP2530	[Ycplac111] *ADH1p-EGO4*	This study	2.2C
pNP2574	[pRS416] *ADH1p-EGO2*	This study	2.2C
pNP2573	[pRS416] *ADH1p-EGO4*	This study	2.2C
Ycplac33	CEN, *URA3*	Gietz, 1988	
pMB1394	[Ycplac33] Tet_{ON}-$GTR1^{Q65L}$ *(GTR1-GTP)*	Binda, 2009	2.2A, B
pMB1395	[Ycplac33] Tet_{ON}-$GTR1^{S20L}$ *(GTR1-GDP)*	Binda, 2009	2.2A, B
pPM1619	[Ycplac33] Tet_{ON}-$GTR2^{Q66L}$ *(GTR2-GTP)*	This study	2.2A, B
pPM1620	[Ycplac33] Tet_{ON}-$GTR2^{S23L}$ *(GTR2-GDP)*	This study	2.2A, B
pMP1639	[pRS415] *GTR1p-GTR1-GFP*	Binda, 2009	2.3
pMP1642	[pRS415] *GTR2p-GTR2-GFP*	Binda, 2009	2.3
pFLJ1895	[pRS416] *CYC1p-HHF2-Tdimer2(12)*	This study	2.3
pNP2441	[pRS413] *EGO4p-EGO4-GFP*	This study	2.4A
pNP2442	[pRS413] *EGO2p-EGO2-GFP*	This study	2.4A
pAI-Alg5	*2μ, ADH1-HA-NUBI, TRP1*	Dualsystems	2.4B
pDL2-Alg5	*2μ, ADH1-HA-NUBG, TRP1*	Dualsystems	2.4B
pCabWT	CEN, *CYC1-CUB-LEXA, LEU2*	Dualsystems	2.4B
pFLJ2393	[pCabWT] *CYC1-EGO4-CUB-LEXA*	This study	2.4B
pFLJ2394	[pCabWT] *CYC1-EGO5-CUB-LEXA*	This study	2.4B
pPR3-N	*2μ, CYC1-NUBG-HA, TRP1*	Dualsystems	2.4B
pNP1689	[pPR3-N] *CYC1-NUBG-HA-GTR1*	Binda, 2009	2.4B
pNP1690	[pPR3-N] $CYC1\text{-}NUBG\text{-}HA\text{-}GTR1^{S20L}$	Binda, 2009	2.4B
pNP1691	[pPR3-N] $CYC1\text{-}NUBG\text{-}HA\text{-}GTR1^{Q65L}$	Binda, 2009	2.4B
pNP1692	[pPR3-N] *CYC1-NUBG-HA-GTR2*	Binda, 2009	2.4B
pNP1693	[pPR3-N] $CYC1\text{-}NUBG\text{-}HA\text{-}GTR2^{S23L}$	Binda, 2009	2.4B
pNP1694	[pPR3-N] $CYC1\text{-}NUBG\text{-}HA\text{-}GTR2^{Q65L}$	Binda, 2009	2.4B
pNP1696	[pPR3-N] *CYC1-NUBG-HA-EGO1*	Binda, 2009	2.4B
pFLJ2221	[pPR3-N] *CYC1-NUBG-HA-EGO3*	Zhang, 2012	2.4B
pNP2564	[pET15b] HIS_6*-EGO1,EGO2,EGO3, EGO4*	This study	2.4C, D

Table 5. Strains used in the chapter III

Strain	Genotype	Source	Figure
YL515	[BY4741/2]*MATα*; *his3Δ1, leu2Δ0, ura3Δ0*	Binda, 2009	3.1; 3.3; 3.4
YL516	[BY4741/2]*MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0*	Binda, 2009	3.5
MP06-8B	[YL515] *MATα*; *gtr1Δ::kanMX4, gtr2Δ::kanMX4*	Binda, 2009	3.4A
NP2259	[BY4741] *MAT***a**; *spe1Δ::KanMX*	Euroscarf	3.5
NP2260	[BY4741] *MAT***a**; *spe2Δ::KanMX*	Euroscarf	3.5
NP2261	[BY4741] *MAT***a**; *spe3Δ::KanMX*	Euroscarf	3.5
NP2262	[BY4741] *MAT***a**; *spe4Δ::KanMX*	Euroscarf	3.5
NP2263	[BY4741] *MAT***a**; *fms1Δ::KanMX*	Euroscarf	3.5
NP2264	[BY4741] *MAT***a**; *paa1Δ::KanMX*	Euroscarf	3.5

Table 6. Plasmids used in the chapter III

Plasmid	Description	Source	Figure
pRS413	CEN, *HIS3*	Brachman, 1998	3.1; 3.3; 3.4B; 3.5
pRS415	CEN, *LEU2*	Brachman, 1998	3.4A
pRS416	CEN, *URA3*	Brachman, 1998	3.1; 3.3; 3.4; 3.5
pJU1064	[pRS413] SCH9^{T570A}-HA$_5$	Urban, 2007	3.4A
pJU1058	[pRS415] SCH9^{T570A}-HA$_5$	Urban, 2007	3.1; 3.3; 3.5
pJU1030	[pRS416] SCH9^{T570A}-HA$_5$	Urban, 2007	3.4B
Ycplac33	CEN, *URA3*	Gietz, 1988	
pMB1393	[YCplac33] *Tet$_{ON}$-GTR1 (GTR1-WT)*	Binda, 2009	3.4A
pMB1394	[Ycplac33] *Tet$_{ON}$-GTR1^{Q65L} (GTR1-GTP)*	Binda, 2009	3.4A
YCplac111	CEN, *LEU2*	Gietz, 1998	
pPM1621	[YCplac111] *Tet$_{ON}$-GTR2 (GTR2-WT)*	Panchaud, 2013	3.4A
pPM1623	[YCplac111] *Tet$_{ON}$-GTR2^{S23L} (GTR2-GDP)*	Bonfils, 2012	3.4A
pTP1485	[pRS315] *HA$_3$-TOR1-A1957V*	Reinke, 2006	3.4B
pTP1486	[pRS315] *HA$_3$-TOR1-I1954V*	Reinke, 2006	3.4B
pMB1379	[YCplac33] *Met15*	This study	3.5

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Appendix

Publications

Amino Acid Deprivation Inhibits TORC1 Through a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr1. (2013)
Panchaud N, Péli-Gulli MP, De Virgilio C.
Science Signaling. 6 (277):ra42

SEACing the GAP that nEGOCiates TORC1 activation: Evolutionary conservation of Rag GTPase regulation. (2013)
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Identification of a Small Molecule Yeast TORC1 Inhibitor with a Multiplex Screen Based on Flow Cytometry. (2012)
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ACS Chemical Biology. 7(4):715-22

CELL BIOLOGY

Amino Acid Deprivation Inhibits TORC1 Through a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr1

Nicolas Panchaud,* Marie-Pierre Péli-Gulli,* Claudio De Virgilio†

The Rag family of guanosine triphosphatases (GTPases) regulates eukaryotic cell growth in response to amino acids by activating the target of rapamycin complex 1 (TORC1). In humans, this pathway is often deregulated in cancer. In yeast, amino acids promote binding of GTP (guanosine 5′-triphosphate) to the Rag family GTPase Gtr1, which, in combination with a GDP (guanosine diphosphate)–bound Gtr2, forms the active, TORC1-stimulating GTPase heterodimer. We identified Iml1, which functioned in a complex with Npr2 and Npr3, as a GAP (GTPase-activating protein) for Gtr1. Upon amino acid deprivation, Iml1 transiently interacted with Gtr1 at the vacuolar membrane to stimulate its intrinsic GTPase activity and consequently decrease the activity of TORC1. Our results delineate a potentially conserved mechanism by which the Iml1, Npr2, and Npr3 orthologous proteins in humans may suppress tumor formation.

INTRODUCTION

The structurally and functionally conserved target of rapamycin complex 1 (TORC1) is a central element of a signaling pathway that integrates various hormonal, growth factor, energy, and nutritional cues to coordinate growth, metabolism, and aging (*1*, *2*). In yeast, activated TORC1 propagates nutrient signals mainly through the AGC protein kinase Sch9 and Tap42, a regulator of type 2A (and type 2A like) protein phosphatases, to favor anabolic processes and inhibit catabolic processes and stress response programs (*3*, *4*). A primordial TORC1 input signal that cannot be compensated for by any other stimulus is provided by amino acids, in particular branched-chain amino acids such as leucine. The mechanism through which TORC1 senses amino acids requires the highly conserved Rag family of guanosine triphosphatases (GTPases), which function in heterodimeric complexes that combine RagA or RagB with RagC or RagD in higher eukaryotes, or Gtr1 with Gtr2 in yeast (*5*–*7*). The Rag or Gtr heterodimers are asymmetrically loaded with GTP (guanosine 5′-triphosphate) and GDP (guanosine diphosphate) and can stimulate TORC1 in response to amino acids when RagA, RagB, or Gtr1 is bound to GTP. The mechanistic details of how amino acids modulate the configuration of the Rag or Gtr heterodimers are still a matter of debate, but likely involve both specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). It has been proposed that lysosomal amino acids, through an inside-out mechanism that requires the vacuolar H^+-ATPase (H^+-translocating adenosine triphosphatase) (v-ATPase) (*8*), stimulate the GEF activity of the Ragulator complex toward RagA or RagB (*9*, *10*). Whether this represents an ancestral mode of the regulation of Rag GTPase activity remains to be determined because yeast cells do not express apparent orthologs of critical components of the Ragulator complex and may promote GTP loading of Gtr1 through the GEF Vam6 (*6*, *11*). Amino acid signaling may also involve leucyl tRNA (transfer RNA) synthetase, which acts as a leucine sensor that promotes the active conformation of the Rag heterodimers (*12*, *13*). A putative GAP that suppresses the activity of RagA, RagB, or Gtr1 after amino acid deprivation has yet to be identified. Here, we report on our discovery that in yeast, Iml1 functions in an Npr2/3-containing complex as a GAP for Gtr1 to decrease the activity of TORC1 and consequently growth after amino acid deprivation.

RESULTS

A genome-wide screen for regulators that inhibit TORC1 in response to amino acid deprivation identified the yeast proteins Npr2 and Npr3 (*14*), which together with Iml1 assemble into a complex that is embedded within the larger, vacuolar membrane associated SEA complex (SEAC) (*15*). The Iml1-Npr2-Npr3 complex has also been proposed to promote autophagy, although independently of TORC1 (*16*). To clarify the role of SEAC in TORC1 regulation, we assessed TORC1 activity in cells lacking individual components of the SEAC, all of which grew normally at 30°C and responded properly to leucine starvation in terms of phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), a sensitive indicator of the presence of uncharged tRNAs that stimulate the eIF2α kinase Gcn2 (*17*) (fig. S1, A and B). Loss of Iml1, Npr2, or Npr3, but not of Sea2, Sea3, or Sea4, resulted in increased TORC1 activity, as assessed by phosphorylation of Sch9 (Fig. 1, A and B). Together with the observation that the concomitant loss either of Npr2 and Npr3 or of Npr2, Npr3, and Iml1 stimulated TORC1 activity to similar extents (Fig. 1C and fig. S2), these data indicate that Npr2, Npr3, and Iml1 share a common biological function in inhibiting TORC1. In contrast, Sea2, Sea3, and Sea4 appeared to redundantly attenuate the TORC1 inhibitory properties of the Iml1-Npr2-Npr3 complex because combined loss of Sea2, Sea3, and Sea4 (Sea2/3/4) decreased TORC1 activity in an Iml1-, Npr2-, or Npr3-dependent manner (Fig. 1C and fig. S2). To further dissect the roles of the individual components of the Iml1-Npr2-Npr3 complex, we overproduced Iml1, Npr2, Npr3, or Npr2 together with Npr3 and examined the corresponding effects on TORC1 activity in wild-type, *iml1*Δ, *npr2*Δ, and *npr3*Δ strains. Overproduction of Iml1 bypassed the requirement for Npr2 or Npr3 to decrease the activity of TORC1, but not vice versa (Fig. 1D). Consistent with these genetic data, Iml1 did not require Npr2/3 or Sea2/3/4 to be recruited to the vacuolar membrane (Fig. 1, E and F), whereas Npr2 and Npr3 depended on each other and on Iml1 for their localization to the vacuolar membrane. This led us to speculate that the

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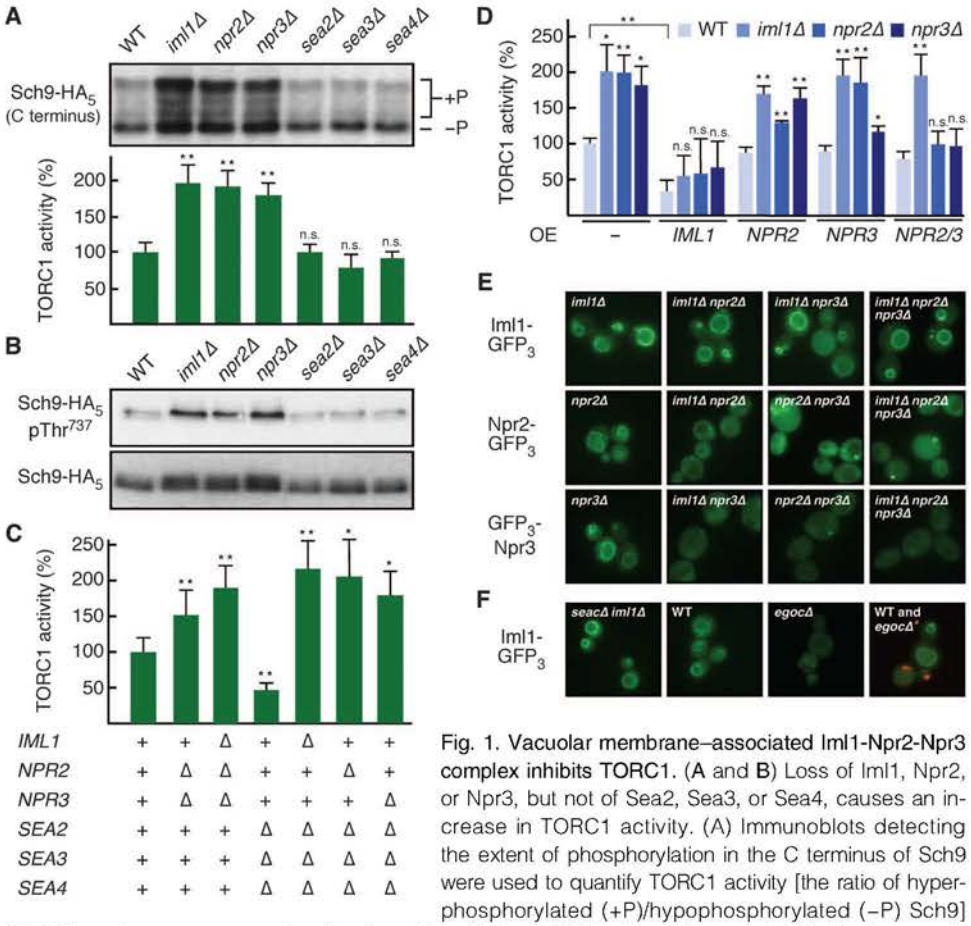

Fig. 1. Vacuolar membrane–associated Iml1-Npr2-Npr3 complex inhibits TORC1. (A and B) Loss of Iml1, Npr2, or Npr3, but not of Sea2, Sea3, or Sea4, causes an increase in TORC1 activity. (A) Immunoblots detecting the extent of phosphorylation in the C terminus of Sch9 were used to quantify TORC1 activity [the ratio of hyperphosphorylated (+P)/hypophosphorylated (−P) Sch9] (*25*). The values were normalized to those for wild-type (WT) cells and presented in the bar graph as means ± SD (n = 3 independent experiments). (B) Similar results for TORC1 activities (ratio of pThr^{737}/total full- length Sch9) were obtained by using specific antibodies recognizing the phosphorylated Thr^{737} (pThr^{737}) of Sch9 (*26*), a major TORC1 target in yeast (*25*). One representative immunoblot from three independent experiments is shown. (C and D) Effects on TORC1 activity of various combinations of individual deletions (Δ) of SEAC subunit–encoding genes (C) and of overexpression (OE) of *IML1* or of *NPR2* and *NPR3* (individually or in combination) in different mutant backgrounds (D). Data are means ± SD from three independent experiments. (E and F) WT or mutant strains expressing the indicated functional green fluorescent protein (GFP) fusion proteins from their own promoter were analyzed by fluorescence microscopy during exponential growth. (F) *egoc*Δ and *seac*Δ denote the *gtr1/2*Δ, *ego1*Δ, *ego3*Δ, and *sea2–4*Δ mutants, respectively. WT and *egoc*Δ* (expressing the nuclear marker Hhf2-RFP) cells were mixed to directly compare Iml1-GFP$_3$ signals at the vacuolar membrane. Representative images from three independent experiments are shown. $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant, compared to the respective WT control using Student's t test (P values are Holm-Bonferroni–adjusted).

functional roles within the Iml1-Npr2-Npr3 complex may be partitioned into a catalytic role for Iml1 and structural and/or regulatory roles for Npr2 or Npr3.

Our cell biological analyses revealed that Iml1 required the presence of the EGO complex [constituting Gtr1 and Gtr2 and their vacuolar membrane anchors Ego1 and Ego3 (*18*)] to be efficiently localized at the vacuolar membrane (Fig. 1F). This suggested that Iml1 may regulate TORC1 through the Gtr1-Gtr2 heterodimer. Consistent with this idea, the absence of Gtr1 or Gtr2 (or of the TORC1 subunit Tco89; Fig. 2A) or the expression of signaling-compromised Gtr1^{S20L} (which has low affinity for nucleotides) or GTP-locked Gtr2^{Q66L} (Fig. 2B) prevented the increase in activation of TORC1 in *iml1*Δ cells. Increased TORC1 activation in *npr2*Δ and *npr3*Δ cells was also dependent on Gtr1 (Fig. 2A). Moreover, expression of the GTP-locked, signaling-competent Gtr1^{Q65L} allele and loss of Iml1, both individually and in combination, stimulated TORC1 to a similar extent, indicating that Iml1 may in fact specifically function upstream of and inhibit Gtr1 (Fig. 2B). Two additional observations support this notion: (i) TORC1 activity remained sensitive to loss or overproduction of Iml1 in the presence of Gtr2^{S23L}, which has low affinity for nucleotides (Fig. 2, B and C), but (ii) was largely refractory to Iml1 overproduction in Gtr1^{Q65L}- or Gtr1^{S20L}-expressing cells (Fig. 2C). Last, as predicted by a model in which the Iml1-Npr2-Npr3 complex decreases the activity of Gtr1 after amino acid deprivation, loss of Iml1, or of Npr2 and Npr3 (individually or in combination), like overexpression of Gtr1^{Q65L}, rendered TORC1 activity partially resistant to leucine starvation (Fig. 2D and fig. S3).

Our genetic epistasis analyses led us to examine whether Iml1 interacted with Gtr1 in cells. Iml1 specifically bound Gtr1 in the presence, but substantially less in the absence, of Npr2 and Npr3 (Fig. 3A and fig. S4). Moreover, although GFP-fused variants of Iml1, Npr2, and Npr3, like EGOC and TORC1 (*6*), all localized to the vacuolar membrane both before and after leucine deprivation (Fig. 3B), bimolecular fluorescence complementation (BiFC) analysis revealed that leucine deprivation stimulated the Iml1-Gtr1 interaction specifically at the vacuolar membrane (Fig. 3C). This was also readily observable in coimmunoprecipitation experiments in which leucine deprivation transiently stimulated the Iml1-Gtr1 interaction, but not the constitutively strong Iml1-Gtr1^{Q65L} interaction (Fig. 3D). To explore whether Iml1 regulated the GTP-loading status of Gtr1 in cells, we made use of the fact that the TORC1 subunit Kog1 binds preferentially the GTP-bound form of Gtr1 (*6*); hence, the amount of Gtr1-associated Kog1 can be used as a proxy for the relative amount of Gtr1^{GTP} within cells. We observed that Iml1 overproduction reduced the Kog1-Gtr1 interaction, but not the Kog1-Gtr1^{Q65L} interaction (Fig. 3E), which implicates Iml1 as a potential GAP for Gtr1. In accordance with this notion, the binding of purified Iml1 to Gtr1 was enhanced by the presence of the nonhydrolyzable GTP analog GTPγS or of GDP-AlF$_x$, which is a structural mimic of the transition state in the hydrolysis reaction by GTPases (Fig. 3F) (*19*) and identifies interactions of GTPases with their cognate GAPs (*20*).

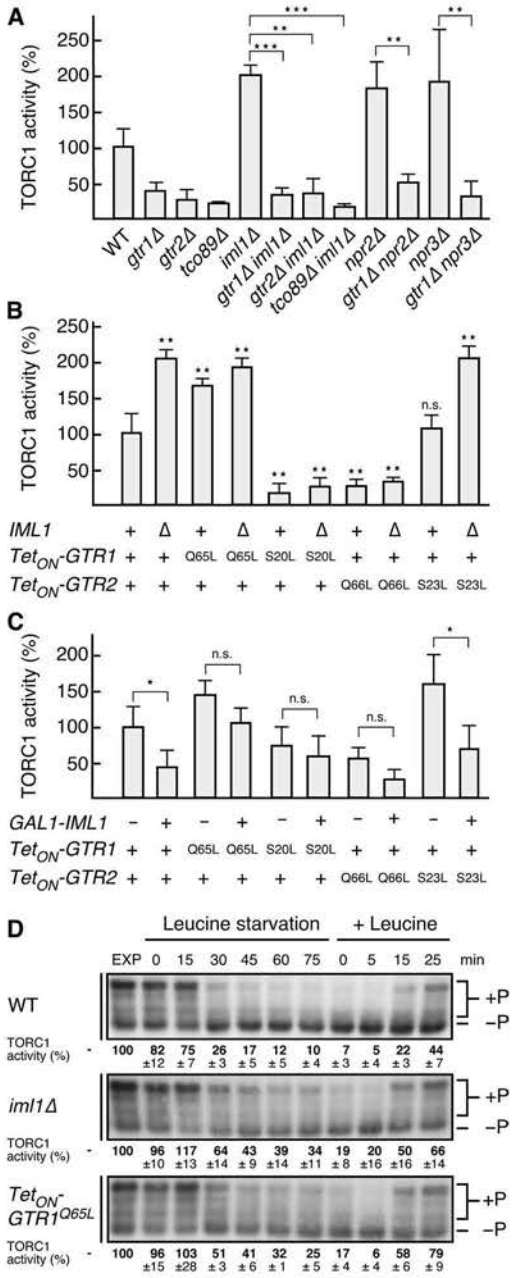

Fig. 2. The Iml1-Npr2-Npr3 complex controls TORC1 activity through the Rag GTPase heterodimer Gtr1-Gtr2. (A) Increased TORC1 activity observed in the absence of Iml1, Npr2, or Npr3 requires the presence of Gtr1 or Gtr2 (means ± SD from three independent experiments). (B and C) Effects on TORC1 activity of loss (B) and overproduction of Iml1 (C) in the presence of overexpressed Gtr1-Gtr2, Gtr1^{Q65L}-Gtr2, Gtr1^{S20L}-Gtr2, Gtr1-Gtr2^{Q66L}, or Gtr1-Gtr2^{S23L} heterodimers (means ± SD from three independent experiments). (D) Loss of Iml1 or expression of Gtr1^{Q65L} renders TORC1 activity partially insensitive to leucine starvation (assayed as in Fig. 1A). Numbers are means ± SD from three independent experiments; one representative immunoblot is shown. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s., not significant, compared to the indicated control in (A) and (C) or to WT in (B) using Student's *t* test (*P* values are Holm-Bonferroni–adjusted).

To verify our assumption that Iml1 has GAP activity toward Gtr1, we performed in vitro GAP assays with purified Iml1 and Gtr1 proteins. In a concentration-dependent manner, Iml1 stimulated the rate of GTP hydrolysis by Gtr1 to a greater extent than that by Gtr2 or the unrelated Rho GTPase Cdc42 (Fig. 4, A and B). Analyses of truncated Iml1 versions further allowed us to specify a conserved domain in Iml1 that was required for proper TORC1 inhibition (Fig. 4C) and was sufficient to stimulate the GTP hydrolysis rate by Gtr1 in vitro (Fig. 4D). In single-turnover GAP assays, this domain, like full-length Iml1, substantially accelerated the catalytic rate of Gtr1-mediated GTP hydrolysis and was therefore coined the Iml1 GAP domain (Iml1^{GAP}; Fig. 4, C and E). GAPs often supply a catalytic amino acid residue such as an arginine (Arg), glutamine (Gln), or aspartate (Asp) into the active site of their GTPases (*19*), which prompted us to carry out an alanine scanning approach. We identified Arg^{943}, which is located within Iml1^{GAP} (Fig. 4C), as critical for the GAP activity of Iml1 in vitro (Fig. 4, D and E). The R943A mutation partially, but significantly, reduced the TORC1 inhibitory function of Iml1 in cells (figs. S3 and S5, A and B). Together with the observation that GDP-AlF$_x$ promoted the in vitro interaction of Gtr1 with wild-type Iml1 and Iml1^{R943A} to the same extent (fig. S6), these data suggest that Arg^{943} is a catalytically, rather than structurally, important residue within the Gtr1 GAP Iml1. However, detailed structural analyses will be required to assess whether Arg^{943} in Iml1 interacts with the catalytic domain of Gtr1. Although Gtr1-Gtr2^{Q66L} or Gtr1-Gtr2^{S23L} heterodimer formation per se stimulated the intrinsic GTPase activity of Gtr1 16- or 128-fold, respectively, Iml1 exhibited GAP activity toward both monomeric and heterodimeric Gtr1 in a comparable range (Fig. 4F). Thus, Gtr1-mediated GTP hydrolysis in Gtr GTPase heterodimers appears to be controlled synergistically by both the GDP/GTP loading status of Gtr2 and the activity of Iml1. Finally, supporting the evolutionary conservation of our findings, we found that the human Iml1 ortholog DEPDC5 could partially rescue the TORC1 inhibition defect in *iml1*Δ cells (Fig. 4G).

DISCUSSION

In association with Npr2 and Npr3, Iml1 functions as a GAP for Gtr1 to restrain it from activating TORC1 specifically in response to amino acid deprivation. Iml1, Npr2, and Npr3 have orthologs in humans (DEPDC5, NPRL2, and NPRL3, respectively), of which NPRL2 has previously been classified as a suppressor of various tumors (*21*–*23*). In addition, the identification of overlapping homozygous deletions encompassing *DEPDC5* (and two other genes) in two cases of glioblastoma suggests that loss of DEPDC5 may contribute to the development of cancer (*24*). Because DEPDC5 could partially complement the loss of Iml1 in yeast, we speculate that the Iml1-Npr2-Npr3 complex may play an evolutionarily conserved role in suppressing Rag-mediated activation of TORC1, thereby contributing to the suppression of human tumor formation.

MATERIALS AND METHODS

Strains, growth conditions, and plasmids

Unless stated otherwise, prototrophic strains were pregrown overnight in synthetic dropout medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mix, and 2% glucose). Before each experiment, cells were diluted to an OD_{600} (optical density at 600 nm) of 0.2 and further grown at 30°C 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 in synthetic dropout medium with leucine (0.37 mg/ml), filtered, washed twice, and resuspended in same medium devoid of leucine. For galactose induction, precultures were grown

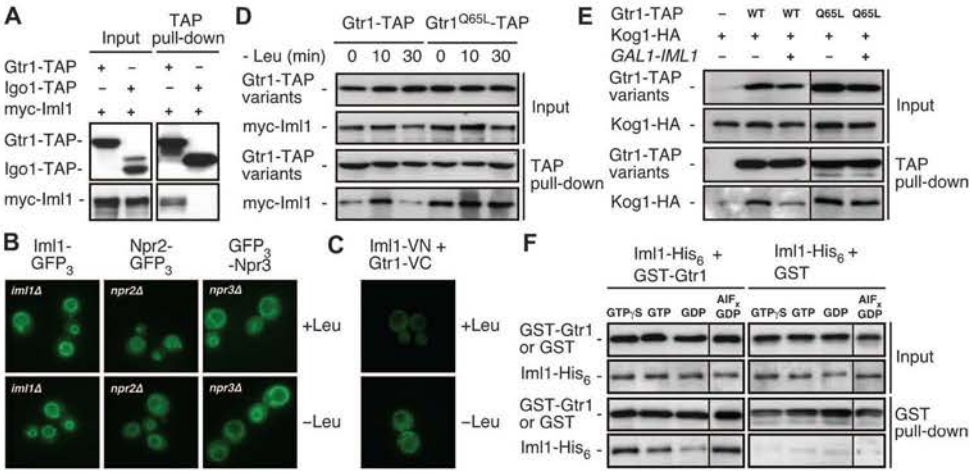

Fig. 3. Leucine inhibits the interaction between Iml1 and Gtr1 at the vacuolar membrane. (A) In exponentially growing WT cells, myc-Iml1 physically interacts with Gtr1-TAP, but not with the control fusion protein Igo1-TAP. Lysates (input) of cells expressing the indicated fusion proteins and TAP pull-down fractions were analyzed by immunoblotting with anti-TAP or anti-myc antibodies. One representative immunoblot from three independent experiments is shown. (B and C) Leucine deprivation does not change the vacuolar membrane localization of Iml1-GFP_3, Npr2-GFP_3, and GFP_3-Npr3 (B), but stimulates the interaction between Gtr1 and Iml1 at the vacuolar membrane as assayed through BiFC (C), which allows detection of protein-protein interactions in cells due to reconstitution of the fluorescent Venus protein (*29*). VN and VC denote N-terminal and C-terminal fragments of Venus, respectively. Representative images are shown from three independent experiments. (D) TAP pull-down analyses indicate that the Iml1-Gtr1 interaction is transiently stimulated after leucine starvation, whereas Iml1 constitutively binds $Gtr1^{Q65L}$. (E) The Gtr1-Kog1 interaction, but not the $Gtr1^{Q65L}$-Kog1 interaction, is reduced when *IML1* is overexpressed from the *GAL1* promoter. (F) Purified Iml1-His_6 preferentially binds to bacterially expressed GST-Gtr1 preloaded with GTPγS, GTP, or AlF_x (AlF_3 or AlF_4^-) plus GDP. Data from (D) to (F) are representative immunoblots from three or more independent experiments.

in synthetic medium (SRaf; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mix, 2% raffinose, and 0.1% sucrose). Cells were then diluted to an OD_{600} of 0.2 and further grown in SRaf supplemented with 2% galactose until they reached an OD_{600} of 0.8. Expression of genes under the control of the Tet_{ON} promoter was induced by adding doxycycline (5 μg/ml) to specified medium. The *S. cerevisiae* strains and plasmids used in this study are listed in tables S1 and S2, respectively.

TORC1 activity assays

TORC1 activity was quantified by assessing the phosphorylation of the C-terminal part of hemagglutinin (HA)–tagged $Sch9^{T570}$, which contains at least five bona fide TORC1 phosphorylation sites, as described previously (*6*, *25*). Briefly, after chemical cleavage with NTCB (2-nitro-5-thiocyanatobenzoic acid), extracts were separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE), and membranes were probed with anti-HA antibodies (12CA5) and anti-mouse immunoglobulin G (IgG) antibodies coupled to horseradish peroxidase (HRP) (Bio-Rad). Alternatively (in Fig. 1B), TORC1 activity was estimated as the ratio between the phosphorylation on Thr^{737} of full-length Sch9-HA_5 compared to the total abundance of Sch9-HA_5 (using phosphospecific anti–pThr^{737}-Sch9 and 12CA5 antibodies, respectively) as previously described (*26*).

Coimmunoprecipitation

Yeast cells expressing the indicated fusion proteins were harvested by filtration. Filters were immediately snap-frozen in liquid nitrogen and stored at −80°C. Cells were resuspended in lysis buffer [50 mM Hepes/KOH (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 0.2% NP-40 (for myc-Iml1) or 0.5% NP-40 (for Kog1-HA), protease and phosphatase inhibitor cocktails (Roche)] and lysed with glass beads using the Precellys cell disruptor. Lysates were diluted in the same lysis buffer (for myc-Iml1) or a buffer devoid of NP-40 (for Kog1-HA) and clarified by two successive centrifugations for 10 min at 13,000 rpm. For input samples, aliquots of cleared lysates were concentrated by precipitation with ice-cold acetone, resuspended in 6× concentrated loading buffer, and denatured for 10 min at 95°C. For coimmunoprecipitations, cleared lysates were incubated for 2 hours at 4°C with prewashed IgG Sepharose beads (GE Healthcare). After three washes with wash buffer (same as lysis buffer for myc-Iml1 or containing 450 mM NaCl for Kog1-HA), beads were resuspended in 6× concentrated loading buffer and denatured for 10 min at 95°C. Inputs (25 μg) and pull-down samples (2000 μg for myc-Iml1, 500 μg for Kog1-HA, and 125 or 67.5 μg for Gtr1-TAP) were analyzed by SDS-PAGE immunoblot with anti-myc (9E10; Santa Cruz Biotechnology), anti-HA (HA.11; Covance), and anti-TAP (Open Biosystems) antibodies together with light chain–specific anti-mouse or anti-rabbit HRP-conjugated antibodies (Jackson ImmunoResearch).

Protein purification

Glutathione *S*-transferase (GST)–Gtr1, GST-Gtr2, GST-Gtr2 (S23L or Q66L), Gtr1-His_6, GST-Cdc42, and GST-$Iml1^{GAP}$ were produced in the *Escherichia coli* Rosetta strain after induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 hours at 18°C [GST-Gtr1, GST-Gtr2, and GST-Gtr2 (S23L or Q66L) plus Gtr1-His_6], 30°C (GST-$Iml1^{GAP}$), or 37°C (GST-Cdc42). Cells were collected by centrifugation and lysed with a microfluidizer. Protein fusions were purified with glutathione-Sepharose beads (GE Healthcare), which were washed with buffer A [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol (DTT), 0.1% NP-40, and 0.1 mM GDP] for GST-Gtr1, GST-Gtr2, GST-Gtr2 (S23L or Q66L) plus Gtr1-His_6, and GST-Cdc42, or buffer B [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 1% Triton X-100, and 0.1% Tween 20] for GST-$Iml1^{GAP}$. Proteins were eluted with buffer A plus 10 mM reduced glutathione (without GDP in the case of GST-$Iml1^{GAP}$). Glycerol was added to a final concentration of 20%. Proteins were snap-frozen in liquid nitrogen and stored at −80°C. For the purification of full-length Iml1 and $Iml1^{R943A}$, yeast *iml1*Δ *gtr1*Δ double-mutant cells expressing Iml1- or $Iml1^{R943A}$–His_6–TEV-(tobacco etch virus)-cleavage-site-ProteinA were grown in synthetic dropout medium overnight, diluted to an OD_{600} of 0.2 in YPD (yeast extract, peptone, and dextrose), and further grown to an OD_{600} of 2.0. Cells were then collected by centrifugation and lysed with a planetary micro mill (Pulverisette). Protein fusions were purified with IgG Sepharose beads (GE Healthcare), which were washed with buffer C

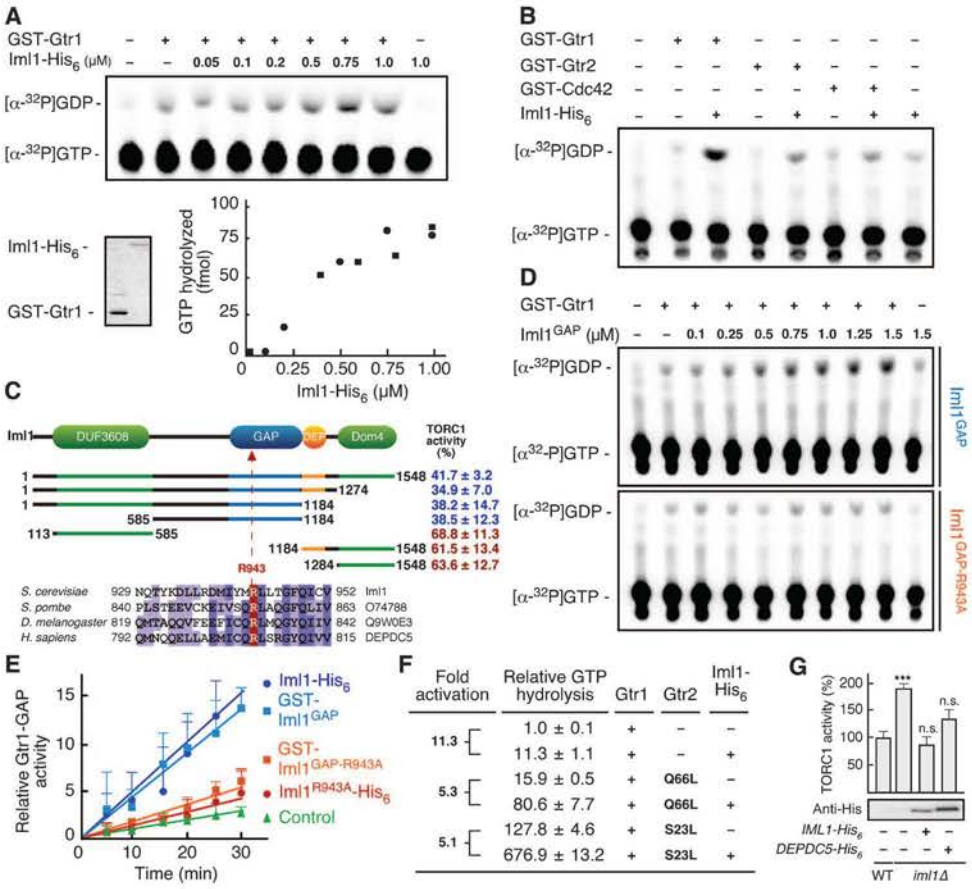

Fig. 4. Iml1 is a GAP for Gtr1. (A) GST-Gtr1 was loaded with [α-^{32}P]GTP, and hydrolysis to [α-^{32}P]GDP was assayed in the absence or presence of increasing concentrations of Iml1-His$_6$. Purified proteins were visualized by Coomassie staining (inserted panel), and the results of the GAP assay were quantified and illustrated graphically. One representative thin-layer chromatography (TLC) autoradiograph and the corresponding quantifications from two independent experiments (squares or circles) are shown. (B) Iml1-His$_6$ activates the intrinsic GTPase activity of Gtr1 to a greater extent than that of Gtr2 or the Rho GTPase Cdc42. One representative TLC autoradiograph from two independent experiments is shown. (C) Schematic representation of the conserved domains within *Saccharomyces cerevisiae* Iml1 and functional analysis of the TORC1 inhibitory activity of the indicated truncated Iml1 variants. The corresponding constructs were overexpressed in WT cells, and TORC1 activities were normalized to the samples containing the empty vector. Red arrow indicates the position of a conserved arginine within the Iml1 GAP domain that was aligned, together with its flanking residues, with the corresponding amino acid sequences of Iml1 orthologs in higher eukaryotes. Data are means ± SD of three independent experiments. (D) The bacterially expressed GAP domain of Iml1 (Iml1^{GAP}), but not Iml1$^{GAP\text{-}R943A}$, activates the GTPase activity of Gtr1. One representative TLC autoradiograph from three independent experiments is shown. (E) Single-turnover GAP assays on Gtr1 with or without (control) the indicated full-length Iml1 or Iml1^{GAP} variants. Data are means ± SD from three independent experiments. (F) Relative GTP hydrolysis (±SD; n = 3 independent experiments) by the indicated combinations of Gtr GTPases (with or without Iml1-His$_6$; normalized to the one of Gtr1 without Iml1-His$_6$). (G) Human DEPDC5 partially complements the TORC1 inhibition defect in *iml1Δ* cells. TORC1 activities were assayed as in Fig. 1A. Fusion protein expression was confirmed by immunoblot analysis. ***$P < 0.001$; n.s., not significant, compared to the respective WT control using Student's t test (P values are Holm-Bonferroni–adjusted). Data are means ± SD from three independent experiments.

[20 mM Hepes (pH 7.4), 300 mM NaCl, 110 mM potassium acetate (KOAc), 2 mM $MgCl_2$, 0.1% Tween 20, 1% Triton X-100, 1 mM DTT] (*15*). After overnight TEV cleavage, a second purification step was performed with Ni-NTA agarose beads (Qiagen), which were washed with buffer C plus 10 mM imidazole. Proteins were eluted with buffer D [20 mM Hepes (pH 7.4), 75 mM NaCl, 110 mM KOAc, 2 mM $MgCl_2$, and 300 mM imidazole]. Glycerol was added to a final concentration of 20%. Proteins were snap-frozen in liquid nitrogen and stored at −80°C.

In vitro Gtr1-Iml1 binding assays

Purified GST-Gtr1 or GST alone (100 nM final) was incubated for 30 min at room temperature in loading buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1 mM DTT] in the presence of either GTPγS (100 μM final), GTP (1 mM final), GDP (1 mM final), or GDP + AlF_x (1 mM GDP, 2 mM $AlCl_3$, and 20 mM NaF final concentration). Subsequently, purified Iml1-His$_6$ (100 nM) and $MgCl_2$ (10 mM) were added, and the mix was incubated for 1 hour at 4°C. Pull-down experiments were performed with glutathione-Sepharose beads (Qiagen), which were washed with loading buffer containing 10 mM $MgCl_2$ (or 10 mM $MgCl_2$, 2 mM $AlCl_3$, and 20 mM NaF; GDP plus AlF_x). Finally, beads were resuspended in 2× Laemmli buffer and boiled, and the supernatants were used for SDS-PAGE immunoblot analyses.

GTP hydrolysis assays

GAP assays were performed essentially as previously described (*27*, *28*). Briefly, GTPases (100 nM) were incubated for 30 min at room temperature in loading buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1 mM DTT] in the presence of 40 nM [α-^{32}P]GTP (Hartman Analytic, 3000 Ci/mmol). Full-length Iml1-His$_6$/Iml1^{R943A}-His6 or GST-Iml1^{GAP}/GST-Iml1$^{GAP\text{-}R943A}$ was then added to the mixtures (at the indicated concentrations), together with 10 mM $MgCl_2$, to initialize the reactions. After 20 min of incubation at room temperature, reactions were stopped by addition of elution buffer (1% SDS, 25 mM EDTA, 5 mM GDP, and 5 mM GTP) and heating for 2 min at 65°C. [α-^{32}P]GTP and [α-^{32}P]GDP were separated by TLC on PEI Cellulose F plates (Merck) with buffer containing 1 M acetic acid and 0.8 M LiCl. Results were visualized with a phosphorimager and quantified with ImageQuant. Single-turnover GAP assays were performed with Iml1-His$_6$ (200 nM), Iml1^{R943A}-His$_6$ (200 nM), GST-Iml1^{GAP} (1 μM), or GST-Iml1$^{GAP\text{-}R943A}$ (1 μM) as described above, except that 1.7 mM unlabeled GTP was added simultaneously with $MgCl_2$.

SUPPLEMENTARY MATERIALS

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Fig. S1. Loss of individual SEAC subunits does not substantially affect the growth of cells at 30°C or eIF2α phosphorylation after leucine starvation.

Fig. S2. Effects on TORC1 activity of various combinations of individual deletions of SEAC subunit-encoding genes.

Fig. S3. Loss of Iml1-Npr2-Npr3 complex subunits, individually or in combination, or expression of $Gtr1^{Q65L}$ or of $Iml1^{R943A}$ renders TORC1 activity partially insensitive to leucine starvation.

Fig. S4. Iml1 physically interacts with Gtr1-TAP in the presence, but substantially less in the absence, of Npr2 and Npr3.

Fig. S5. The conserved residue Arg^{943} in Iml1 is functionally important.

Fig. S6. Purified Iml1-His_6 and $Iml1^{R943A}$-His_6 preferentially bind bacterially expressed GST-Gtr1 preloaded with GDP plus AlF_x.

Table S1. Strains used in this study.

Table S2. Plasmids used in this study.

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Acknowledgments: We thank F. Jaquier and M. Jaquenoud for technical assistance, T. Maeda for anti-$pThr^{737}$-Sch9 antibodies, D. Kressler and R. Loewith for plasmids, and L.-F. Bersier and S. Bacher for advice regarding statistical analyses. **Funding:** This research was supported by the Canton of Fribourg and the Swiss National Science Foundation (C.D.V.). **Author contributions:** N.P. and M.-P.P.-G. performed the experiments. All authors contributed to the experimental design and data analysis and commented on the manuscript, which was written by C.D.V. **Competing interests:** The authors declare that they have no competing interests.

Submitted 28 February 2013
Accepted 12 April 2013
Final Publication 28 May 2013
10.1126/scisignal.2004112
Citation: N. Panchaud, M.-P. Péli-Gulli, C. De Virgilio, Amino acid deprivation inhibits TORC1 through a GTPase-activating protein complex for the Rag family GTPase Gtr1. *Sci. Signal.* **6**, ra42 (2013).

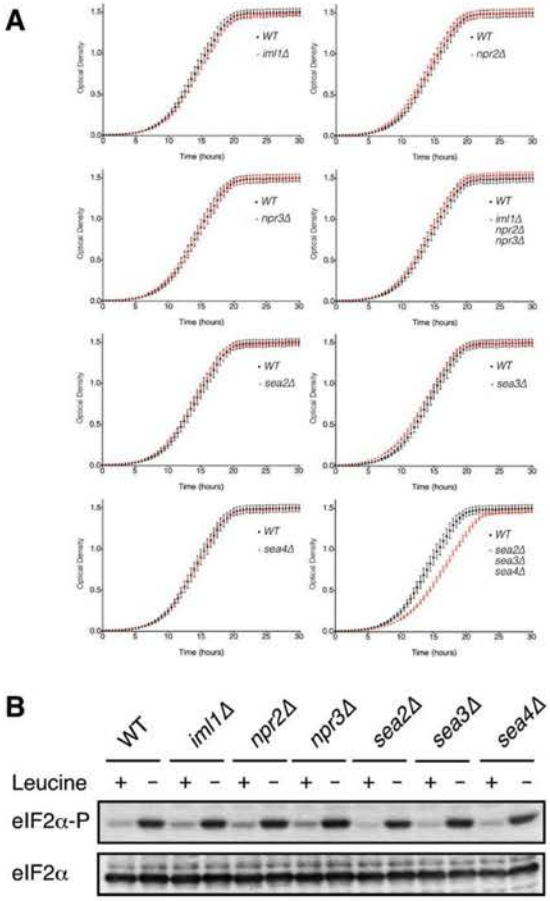

Fig. S1. Loss of individual SEAC subunits does not substantially affect the growth of cells at 30°C or eIF2α phosphorylation after leucine starvation. (**A**) Optical density was measured at 600 nm. As expected based on their intrinsically low TORC1 activity (Fig. 1C), triple *sea2*Δ *sea3*Δ *sea4*Δ mutant cells exhibited a slow growth phenotype (lower panel on the right). Data are means ± S.D. from three independent experiments. (**B**) Phosphorylation of eIF2α (on Ser^{51}; eIF2α-P) was assessed as previously described (*12*) prior to (+) and following (-) a 60-min period of leucine starvation. One representative immunoblot from three independent experiments is shown.

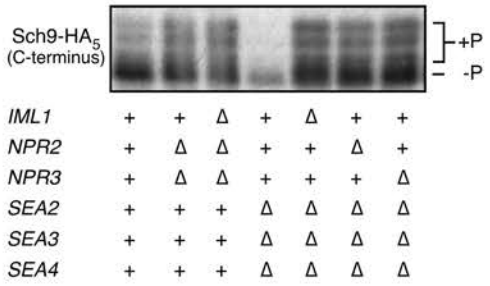

Fig. S2. Effects on TORC1 activity of various combinations of individual deletions of SEAC subunit-encoding genes. Immunoblots (one of 3 is shown) detecting the extent of phosphorylation within the C-

terminus of Sch9 were used to quantify TORC1 activity (the ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9) presented in Fig. 1C.

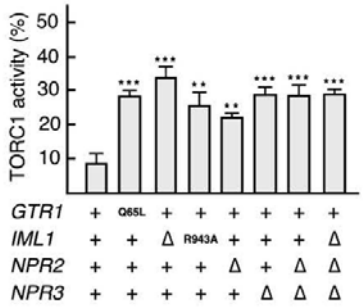

Fig. S3. Loss of Iml1-Npr2-Npr3 complex subunits, individually or in combination, or expression of $Gtr1^{Q65L}$ or of $Iml1^{R943A}$ renders TORC1 activity partially insensitive to leucine starvation. TORC1 activities (means + S.D.; n=3 independent experiments, assayed as in Fig. 1A) were assessed following 30 min of leucine starvation and normalized to the respective value in each strain before leucine starvation. ***P<0.001, **P<0.01 in Student's t-test versus wild-type control (P-values are Holm-Bonferroni adjusted).

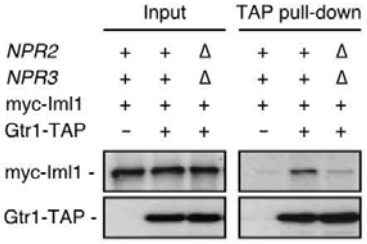

Fig. S4. Iml1 physically interacts with Gtr1-TAP in the presence, but substantially less in the absence, of Npr2 and Npr3. Lysates (Input) from exponentially growing wild-type and *npr2*Δ *npr3*Δ cells expressing the indicated fusion proteins and TAP pull-down fractions were analyzed by immunoblotting using anti-TAP or anti-myc antibodies. One representative immunoblot from three independent experiments is shown.

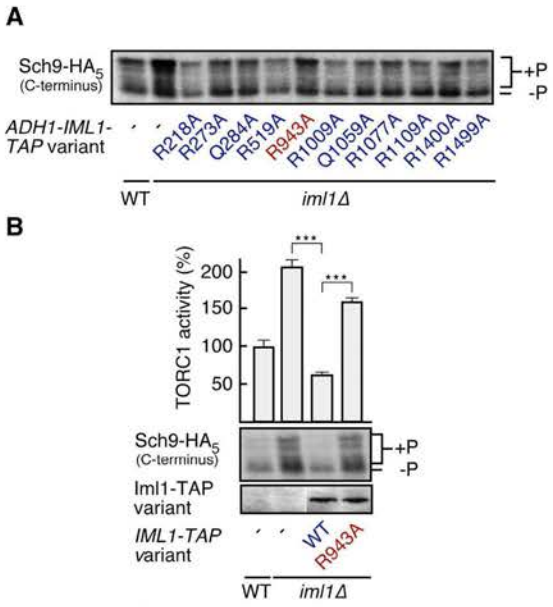

Fig. S5. The conserved residue Arg^{943} in Iml1 is functionally important. **(A)** Alanine scanning of conserved arginine and glutamine residues in Iml1 indicates that Arg^{943} is important for the TORC1-inhibitory activity of overexpressed Iml1-TAP (assayed as in Fig. 1A). One representative immunoblot from two independent experiments is shown . **(B)** Effects of overexpression of Iml1-TAP and $Iml1^{R943A}$-TAP on TORC1 activity were assayed in three independent replicates (means + S.D. are shown). Fusion protein expression was confirmed by immunoblot analysis (lower panel). ***P<0.001, in Student's t-test (P-values are Holm-Bonferroni adjusted).

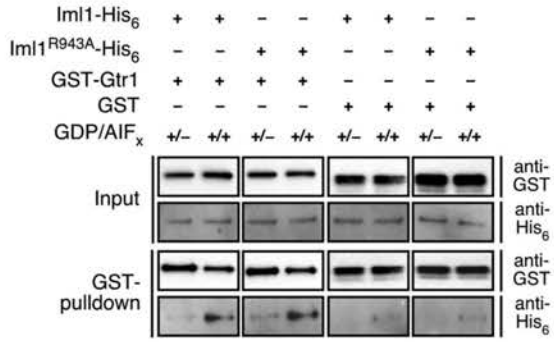

Fig. S6. Purified Iml1-His_6 and $Iml1^{R943A}$-His_6 preferentially bind bacterially expressed GST-Gtr1 preloaded with GDP plus AlF_x. In vitro GST-pulldown experiments were performed with bacterially expressed GST-Gtr1 preloaded with GDP alone (+/-) or GDP plus AlF_x (+/+) and either purified Iml1-His_6 or $Iml1^{R943A}$-His_6. One representative Western blot from two independent experiments is shown.

Cell Cycle 12:18, 2948–2952; September 15, 2013; © 2013 Landes Bioscience

SEACing the GAP that nEGOCiates TORC1 activation

Evolutionary conservation of Rag GTPase regulation

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The target of rapamycin complex 1 (TORC1) regulates eukaryotic cell growth in response to a variety of input signals. In *S. cerevisiae*, amino acids activate TORC1 through the Rag guanosine triphosphatase (GTPase) heterodimer composed of Gtr1 and Gtr2 found together with Ego1 and Ego3 in the EGO complex (EGOC). The GTPase activity of Gtr1 is regulated by the SEA complex (SEAC). Specifically, SEACIT, a SEAC subcomplex containing Iml1, Npr2, and Npr3 functions as a GTPase activator (GAP) for Gtr1 to decrease the activity of TORC1 and, consequently, growth, after amino acid deprivation. Here, we present genetic epistasis data, which show that SEACAT, the other SEAC subcomplex, containing Seh1, Sea2–4, and Sec13, antagonizes the GAP function of SEACIT. Orthologs of EGOC (Ragulator), SEACIT (GATOR1), and SEACAT (GATOR2) are present in higher eukaryotes, highlighting the remarkable conservation, from yeast to man, of Rag GTPase and TORC1 regulation.

Keywords: Rag GTPases, TOR complex 1, EGO complex, SEA complex, amino acid signaling, Iml1-Npr2-Npr3 Rag GTPase GAP complex, GATOR1, GATOR2, Seh1, Sec13

Abbreviations: TORC1, TOR complex 1; EGOC, EGO complex; SEAC, SEA complex

Submitted: 07/12/2013

Accepted: 08/01/2013

http://dx.doi.org/10.4161/cc.26000

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Introduction

The target of rapamycin complex 1 (TORC1) is a structurally and functionally conserved regulator of eukaryotic cell growth that adapts anabolic and catabolic processes in response to a variety of inputs, such as growth factors, cellular stress, energy, and nutrients.[1-4] Amino acids, especially branched-chain amino acids like leucine, represent essential stimuli for TORC1 activation.[5-7] Members of the conserved Rag family of guanosine triphosphatases (GTPases) mediate amino acid signaling to TORC1: in higher eukaryotes, RagA or RagB forms a heterodimer with RagC or RagD, whereas in *S. cerevisiae*, Gtr1 dimerizes with Gtr2. When RagA, RagB, or Gtr1 is bound to GTP, and RagC, RagD, or Gtr2 to GDP, the respective heterodimer is in its active, TORC1-stimulating conformation.[8-11] In mammalian cells, Rag GTPases do not directly activate TORC1, but trigger TORC1 relocalization from the cytoplasm to the limiting membrane of the lysosome, where it can be activated by the GTPase Rheb.[5,10,12] In *S. cerevisiae*, TORC1 remains associated with the limiting membrane of the vacuole (the yeast equivalent to the lysosome) irrespective of the presence or absence of leucine. Moreover, the yeast Rheb ortholog, Rhb1, is likely not required for the regulation of TORC1.[4,9] Thus, the mechanisms by which the Gtr1–Gtr2 heterodimer controls TORC1 function in *S. cerevisiae* remains mysterious.

Gtr and Rag heterodimers are core switches that fulfill their function as part of larger protein complexes. In *S. cerevisiae*, Gtr1-Gtr2 associates with Ego1 and Ego3 to form the EGO complex (EGOC). Ego1 is N-terminally myristoylated and palmitoylated and thus tethers the EGOC to the vacuolar membrane.[9,13-17] Ego3, the precise function of which remains unknown, forms homodimers that, like the C-terminal domains of Gtr1 and Gtr2, are structurally similar to members of the Roadblock/LC7 superfamily of proteins.[15,17] In mammals, Rag GTPase heterodimers associate with the Ego1 equivalent p18 (LAMTOR1), the

Ego3-Ego3-related heterodimer p14-MP1 (LAMTOR2-LAMTOR3),[17] C7orf59 (LAMTOR4), and HBXIP (LAMTOR5), which form the Ragulator complex.[12] Like the EGOC, the Ragulator complex sits on the limiting membrane of the lysosome by virtue of lipidation of p18, which is the only Rag–Ragulator subunit lacking structural resemblance with Roadblock domains (RDs).[5,6,12] The entire pentameric Ragulator complex is proposed to act as the guanine nucleotide exchange factor (GEF) for RagA and RagB.[18] Whether the EGOC possesses similar GEF activity remains questionable, because *S. cerevisiae* cells lack apparent orthologs of HBXIP and C7orf59, and guanine nucleotide exchange on Gtr1 is rather proposed to be stimulated by a Vam6-dependent mechanism.[9] A GTPase-activating protein (GAP) that regulates Rag/Gtr proteins has, until recently, remained elusive.

Recently, subunits of the octameric vacuolar Seh1-associated complex (SEAC) were implicated in negative regulation of TORC1 in yeast.[19-22] In an effort to clarify the relationship between SEAC and TORC1, we discovered in genetic epistasis analyses that the Iml1–Npr2–Npr3 SEAC subcomplex, which we now name SEACIT (for SEAC subcomplex Inhibiting TORC1 signaling), negatively regulates TORC1 through Gtr1 within the EGOC.[23] Moreover, in line with our genetic data, we found that leucine deprivation triggered Iml1 to transiently interact with Gtr1 (in a Npr2- and Npr3-dependent manner) to stimulate its intrinsic GTPase activity. Of note, both Npr2 and Npr3 contain a N-terminal longin domain, the structure of which is closely related to RDs and may serve as platform for Rag GTPases.[24] The GAP activity of SEACIT is conserved, as the orthologous complex in *Drosophila* and human cells (i.e., DEPDC5-Nprl2-Nprl3), coined GATOR1, also acts as a GAP toward RagA and RagB.[25] Intriguingly, various glioblastomas and ovarian cancers contain nonsense or frameshift mutations or truncating deletions in GATOR1-encoding genes, and a number of cancer cell lines with homozygous deletions in *DEPDC5*, *NPRL2*, or *NPRL3* exhibit hyperactive mTORC1 that is insensitive to amino acid deprivation.[25] Since these GATOR1-inactivating mutations also cause hypersensitivity to the TORC1 inhibitor rapamycin in mammalian cells, they may help to predict the therapeutic benefit of clinically approved TORC1 inhibitors in cancer treatments.[25]

In addition to Iml1, Npr2, and Npr3 (SEACIT), the octameric SEAC also contains Sea2, Sea3, Sea4, Seh1, and Sec13, orthologs of the mammalian and *Drosophila* GATOR2 subcomplex proteins WDR24, WDR59, Mios, Seh1L, and Sec13, respectively. These proteins form the other SEAC-subcomplex, which we now name SEACAT (for SEAC subcomplex Activating TORC1 signaling). Except for Sec13, all of the GATOR2 components have been implicated in negative regulation of GATOR1 in higher eukaryotes.[25] Similarly, yeast Sea2, Sea3, and Sea4 antagonize, although redundantly, the SEACIT-mediated TORC1 inhibition.[23] However, roles for yeast Seh1, or either

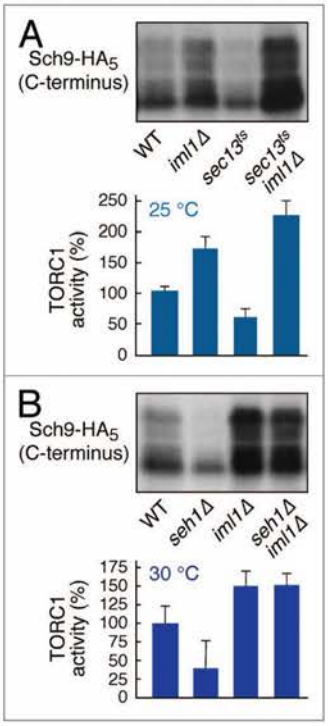

Figure 1. Loss of Iml1 suppresses the TORC1 activation defect in *sec13*^{ts} (**A**) and *seh1*Δ (**B**) cells. Indicated (prototrophic) strains expressing a plasmid-based copy of Sch9^{TS570A}-HA$_5$ were grown exponentially at 25 °C (**A**) or 30 °C (**B**). Immunoblots detecting the level of phosphorylation within the C terminus of Sch9 were used to quantify in vivo TORC1 activity as previously described.[50] Bar graphs refer to the mean ratio (± S.D.) of hyperphosphorylated/hypophosphorylated Sch9 from 3 independent experiments, normalized to the values for wild-type cells.

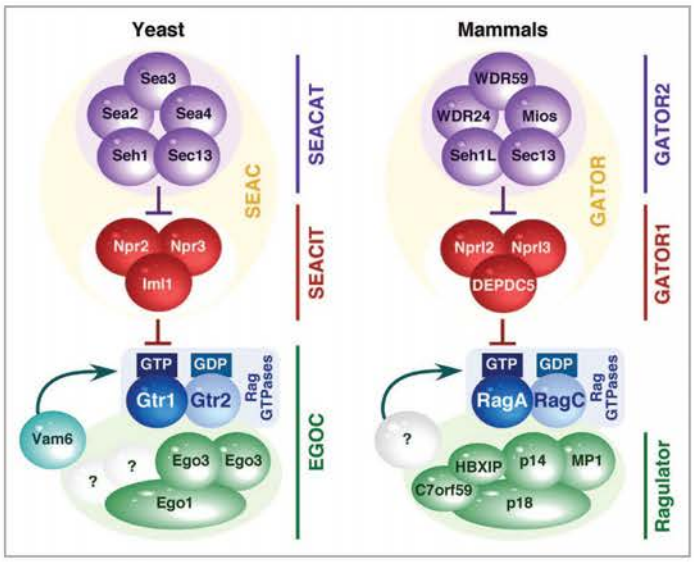

Figure 2. Conserved regulators of the Rag-family GTPases. The yeast SEAC is composed of 2 subcomplexes, SEACIT and SEACAT. SEACAT antagonizes the GAP-function of SEACIT. Vam6 is thought to be the GEF for Gtr1, which resides in the EGOC on the vacuolar membrane. Similarly, the mammalian (and *Drosophila*) GATOR complex is composed of the 2 subcomplexes GATOR1 and GATOR2. GATOR2 antagonizes the GAP-function of GATOR1. Whether or not mammalian Vam6 orthologs (i.e., the TGF-β receptor-associated protein 1 [TRAP1 or TGFBRAP1] and the TRAP1-like protein [TLP], aka hVPS39)[45,46,52-54] act as a RagA/B GEF is unclear, rather the pentameric Ragulator complex, acting downstream of the vacuolar ATPase, is reported to serve this function. For details, please see text.

yeast or metazoan Sec13 upstream of the Rag GTPases are currently not reported.

Results and Discussion

To determine if Sec13, like other SEACAT components, controls TORC1 activity via SEACIT, we assayed TORC1 activity in a temperature-sensitive *sec13*^{ts} (*sec13-1*)[26] mutant. As is shown in **Figure 1A**, the *sec13*^{ts} mutant exhibited significantly reduced TORC1 activity when grown at the permissive temperature of 25 °C. This reduced TORC1 activity matches well with the observation that *sec13-1* is synthetic lethal when combined with a hypomorphic allele of LST8 (i.e., *lst8-1* for lethal with sec-thirteen), which encodes a stimulatory component in TOR-containing complexes.[27,28] Importantly, loss of Iml1 strongly activated TORC1 in both wild-type and *sec13*^{ts} mutant cells. Similarly, we also observed that loss of Seh1 resulted in a significant reduction of TORC1 activity, which was fully suppressed in the absence of Iml1 (**Fig. 1B**). These genetic data therefore support a model in which Sec13 and Seh1, together with the other SEACAT components, promote TORC1 activity through inhibition of the GAP function of SEACIT. These results extend the remarkable evolutionary conservation of TORC1 regulation by Rag GTPases and delineate an inhibitory role for the pentameric SEACAT/GATOR2 subcomplex upstream of the SEACIT/GATOR1 subcomplex (**Fig. 2**).

Curiously, both Sec13 and Seh1 not only function within the SEAC, but also within the nuclear pore complex (NPC) as part of the conserved heptameric Nup84 subcomplex that is essential for the overall architecture of the NPC and consequently the transport of mRNAs and macromolecules (e.g., pre-ribosomes) across the nuclear membrane.[29] Moreover, Sec13 also associates with Sec31 into a heterotetramer, which forms the outer shell of coatmer complex II (COPII) coated vesicles of the secretory pathway that bud off from the endoplasmic reticulum (ER).[30,31] The occurrence of Sec13 and Seh1 in functionally different protein complexes suggests that their 3-dimensional structure, which is characterized, like those of all other SEACAT subunits, by the presence of WD-40 repeats that form β-propellers,[19,21] renders them particularly well suited to serve as building and/or scaffolding blocks within larger protein complexes. Given these observations, it is tempting to speculate that Sec13/Seh1 serve to couple nuclear-to-cytoplasmic mRNA/protein transport or protein secretion to TORC1 control. For instance, compromised nuclear pore function or secretion may tie up or jam Seh1 and/or Sec13, thereby causing reduced SEAC assembly and, consequently, downregulation of TORC1. Interestingly, a genome-scale RNA interference screen by dsRNA reverse-transfection on living *Drosophila* cell microarrays identified nuclear pore components as TORC1 regulators.[32] In a similar vein, alterations in the yeast secretory pathway have also been found to converge on TORC1 regulation. For instance, loss of the Golgi Ca^{2+}/Mn^{2+} ATPase Pmr1 strongly increased the secretion of (heterologous) proteins that transit through the secretory pathway and, based on genetic experiments, also caused TORC1 activation (e.g., *pmr1*Δ suppressed the rapamycin-sensitive phenotype of the *lst8-1* mutation).[33,34] Conversely, addition of the secretory pathway inhibitor tunicamycin and inactivation of the Rab escort protein Mrs6 both strongly inhibited TORC1-dependent phosphorylation of Sch9.[28,35] In sum, these observations lend support to a model in which both NPC function and secretory pathway flux are part of an increasing number of physiological cues (including v-ATPase activity, leucyl-tRNA synthetase function, glutaminolysis-driven production of α-ketoglutarate, glucose and amino acid levels, vesicle trafficking, or actin polarization),[9,36-46] which may converge on Rag GTPase-mediated control of TORC1 (**Fig. 3**). Future studies should therefore aim at deciphering whether any of these cues may fine-tune TORC1 by regulating the GTP loading status of Rag GTPases through the SEACIT/GATOR1 and/or SEACAT/GATOR2 complexes.

Materials and Methods

Growth conditions, strains, and plasmids

Unless stated otherwise, prototrophic strains were pre-grown overnight in synthetic defined dropout (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% [-adenine/-histidine/-leucine/-uracil/-tryptophan] dropout mix, and 2% glucose). For TORC1 activity assays, cells were diluted to an OD_{600} of 0.2 and further grown at 30 °C until they reached an OD_{600} of 0.8. The following isogenic *S. cerevisiae* strains (all wild-type for *LYS2* and *MET15* in the BY4741/2 background)[47] were used in this study: MATα *his3Δ1, leu2Δ0, ura3Δ0* (YL515; WT)[9]; MATα *iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0* (NP04-4C)[23]; MATα *seh1Δ::kanMX,*

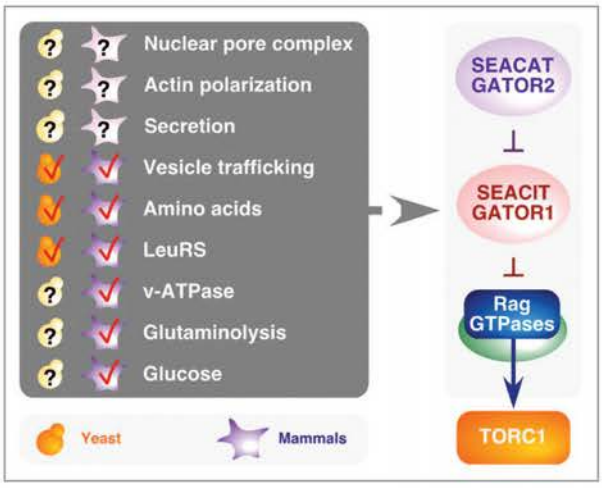

Figure 3. Physiological cues, which may regulate TORC1 through the Rag GTPase module. Red check marks indicate the existence of experimental data supporting (in yeast or mammalian cells) a model in which the respective cue impinges on Rag GTPase regulation (please see text for corresponding references). Currently speculative processes are denoted with a question mark.

his3Δ1, leu2Δ0, ura3Δ0 (MP308-7A); MATα *seh1Δ::kanMX, iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP308-8B); MATα *sec13^{ts}-kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP309-5D); and MATα *sec13^{ts}-kanMX, iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP309-9A). The original *sec13^{ts}* (MAT**a** *sec13-1-kanMX, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*)[48] and *seh1Δ* (MAT**a** *seh1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*)[49] mutants were rendered wild-type for *MET15* by backcrossing with YL515. Sequencing of the *sec13^{ts}* ORF revealed that this allele carries 2 mutations, which change Lys^{44} and Ser^{224} in Sec13 to Glu^{44} and Asp^{224}, respectively. All strains carried the following plasmids: pRS413-Sch9^{T570A}-HA$_5$,[50] pRS415,[51] and pRS416.[51]

TORC1 activity assays

TORC1 activity was determined by quantification of the phosphorylation of the C-terminal part of HA$_5$-tagged Sch9^{T570A}, which contains 5 TORC1 phosphorylation sites, as described previously.[9,50] Briefly, following chemical cleavage with NTCB, extracts were separated by 7.5% SDS-PAGE, and membranes were probed with anti-HA antibodies (12CA5) and anti-mouse IgG antibodies coupled to HRP (Biorad).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Michael Costanzo for strains, Floriane Jaquier for technical assistance, and R Loewith for plasmids and critical and valuable comments on the manuscript. This research was supported by the Canton of Fribourg and the Swiss National Science Foundation (CDV).

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Identification of a Small Molecule Yeast TORC1 Inhibitor with a Multiplex Screen Based on Flow Cytometry

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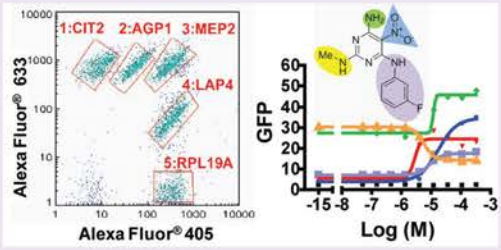
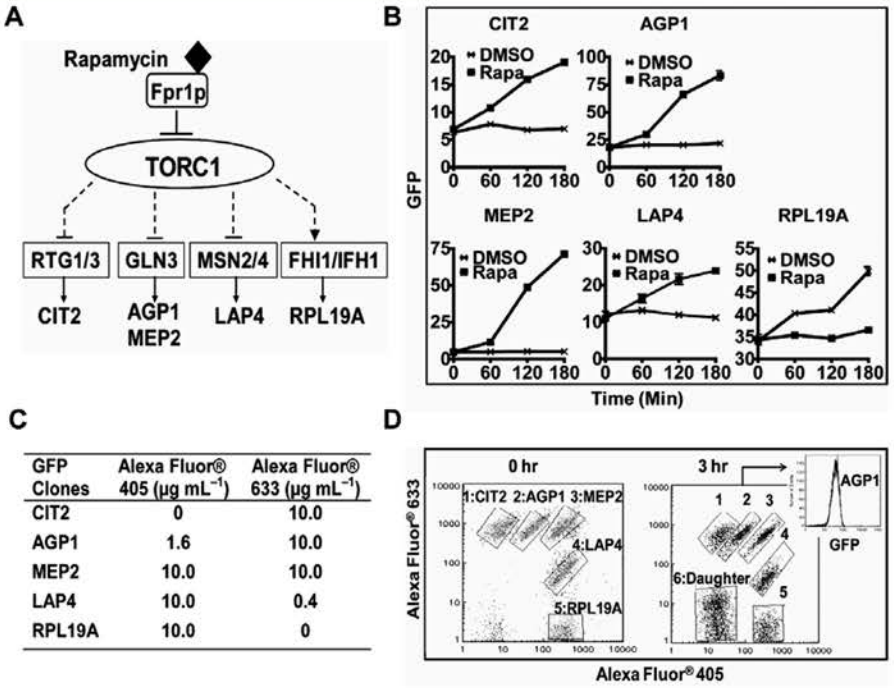
Supporting Information

ABSTRACT: TOR (target of rapamycin) is a serine/threonine kinase, evolutionarily conserved from yeast to human, which functions as a fundamental controller of cell growth. The moderate clinical benefit of rapamycin in mTOR-based therapy of many cancers favors the development of new TOR inhibitors. Here we report a high-throughput flow cytometry multiplexed screen using five GFP-tagged yeast clones that represent the readouts of four branches of the TORC1 signaling pathway in budding yeast. Each GFP-tagged clone was differentially color-coded, and the GFP signal of each clone was measured simultaneously by flow cytometry, which allows rapid prioritization of compounds that likely act through direct modulation of TORC1 or proximal signaling components. A total of 255 compounds were confirmed in dose−response analysis to alter GFP expression in one or more clones. To validate the concept of the high-throughput screen, we have characterized CID 3528206, a small molecule most likely to act on TORC1 as it alters GFP expression in all five GFP clones in a manner analogous to that of rapamycin. We have shown that CID 3528206 inhibited yeast cell growth and that CID 3528206 inhibited TORC1 activity both *in vitro* and *in vivo* with EC_{50}'s of 150 nM and 3.9 μM, respectively. The results of microarray analysis and yeast GFP collection screen further support the notion that CID 3528206 and rapamycin modulate similar cellular pathways. Together, these results indicate that the HTS has identified a potentially useful small molecule for further development of TOR inhibitors.



Target of rapamycin (TOR) proteins are Ser/Thr protein kinases phylogenetically conserved from yeast to man.[1−3] Yeast possesses two TOR proteins that function in two distinct protein complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 is sensitive to rapamycin and promotes protein synthesis and other anabolic processes, while inhibiting autophagy and other catabolic and stress-response processes.[3] TORC2 is largely insensitive to rapamycin and appears to regulate spatial aspects of growth, such as cell polarity.[4] While there are currently no known TORC2 specific inhibitors, TORC1 can be specifically inhibited with rapamycin, which has been used to characterize the TORC1 pathway in both mammals and budding yeast.[2] With the aid of rapamycin, the yeast TORC1 pathway has been extensively investigated (see the comprehensive review on yeast TORC1 in ref 3). Numerous distal readouts of the yeast TORC1 pathway and distinct signaling branches that are regulated by TORC1/rapamycin have been identified in budding yeast, including (1) the RTG signaling pathway mediated by Rtg1p/Rtg3p that activates genes required for biosynthesis and homeostasis of glutamate and glutamine;[5−8] (2) the nitrogen-discrimination pathway (NDP) mediated by Gln3p that activates genes

Received: November 4, 2011
Accepted: January 19, 2012
Published: January 19, 2012



GFP Clones	Alexa Fluor® 405 (μg mL^{-1})	Alexa Fluor® 633 (μg mL^{-1})
CIT2	0	10.0
AGP1	1.6	10.0
MEP2	10.0	10.0
LAP4	10.0	0.4
RPL19A	10.0	0

Figure 1. Development of multiplexing strategies for HTFC screen of the yeast TORC1 pathway. (A) Schematic diagram of the yeast TORC1 pathway showing four signaling branches mediated by various transcription factors and the distal readouts. (B) Time-course analysis of GFP expression in rapamycin-responsive GFP clones. GFP clones were treated with 1% DMSO and 0.22 μM rapamycin, respectively, and GFP signal was measured by flow cytometry at hourly intervals up to 3 h. (C, D) Multiplexing strategy. Five GFP clones were diluted to 0.5 OD_{600} and differentially stained with Alexa Fluor 405 and Alexa Fluor 633 dyes according to the staining scheme (C). The multiplex staining was analyzed by flow cytometry (D). Five distinct cell populations were distinguished in the dot plot, and each gate (1−5) represents an individual GFP clone (left panel, 0 h). The multiplex was stable for 3 h under normal yeast growth conditions (right panel, 3 h) with a distinct population of unbarcoded daughter cells observed (gate 6). The GFP histogram of *AGP1* cells (gate 2) is displayed at the upper right, showing no cross-contamination of different clones in the multiplex after 3 h.

enabling cells to import and catabolize poor nitrogen sources under nitrogen limitations;[9,10] (3) the stress-response pathway mediated by Msn2p/Msn4p that regulates the transcription response to a wide range of stressors;[11] and (4) signaling that controls translation, such as ribosomal protein synthesis, translation initiation, and mRNA turnover.[12,13] TORC1 regulates gene expression in these pathways mainly by controlling translocation of the transcription factors. The downstream effectors or substrates that link TORC1 activity to these readouts are not well understood. So far, only Sch9p kinase and Tap42p phosphatase have been identified as direct TORC1 substrates that mediate TOR signaling to its distal readouts.[14,15] More effectors and substrates need to be identified. Moreover, these signaling branches are not independent but rather engage in substantial cross-talk while also interacting with other signaling pathways,[16−18] thus constituting a complicated regulatory network. Therefore, there is an ongoing need to identify novel components and mechanisms in the TORC1 pathway as well as to isolate new chemical probes to delineate the TORC1 pathway.

The mammalian TOR cognate, mTOR has emerged as a therapeutic cancer target due to its central roles in controlling cell growth.[1] Rapamycin (or its analogues) is a first generation TOR inhibitor that has shown promising results in preclinical pharmacological studies but has not lived up to expectations in clinical trials.[1,19,20] New mTOR inhibitors or novel chemicals that act in concert with rapamycin would be valuable.[21,22] Although more potent ATP-competitive mTOR inhibitors that target both mTORC1 and mTORC2 have been developed,[23−25] small molecules that selectively and potently inhibit either TORC1 or TORC2 are lacking. These molecules are anticipated as the new generation of TOR inhibitors and are likely suitable for unveiling therapeutically relevant mechanisms.[20]

Budding yeast has been a useful system for high-throughput screening (HTS) and for drug target identification and mechanism discovery.[26−28] More importantly, molecules identified from yeast screens have potential for translation into higher organisms.[22,29] Flow cytometry is a versatile high-speed cell analysis method for proteomics and systems biology.[30] HT flow cytometry (HTFC), such as HyperCyt, enables the processing of 96- or 384-well plates in as little as 3 or 12 min, respectively. It is therefore well suited for large-scale cell screening and selection applications,[31−34] such as budding yeast in suspension cell culture. Taking advantage of the yeast GFP collection, which consists of 4,159 GFP-tagged ORFs comprising 75% of the yeast proteome,[35] we identified rapamycin-responsive GFP clones and conducted a multiplexed HTFC screen to search for compounds that alter GFP expression in five rapamycin-responsive GFP clones. These five GFP clones represent the readouts of four branches of the TORC1 signaling pathway (Figure 1A) and allow evaluation of compound activity on multiple branches simultaneously. We sought to identify molecules that functionally mimic rapamycin

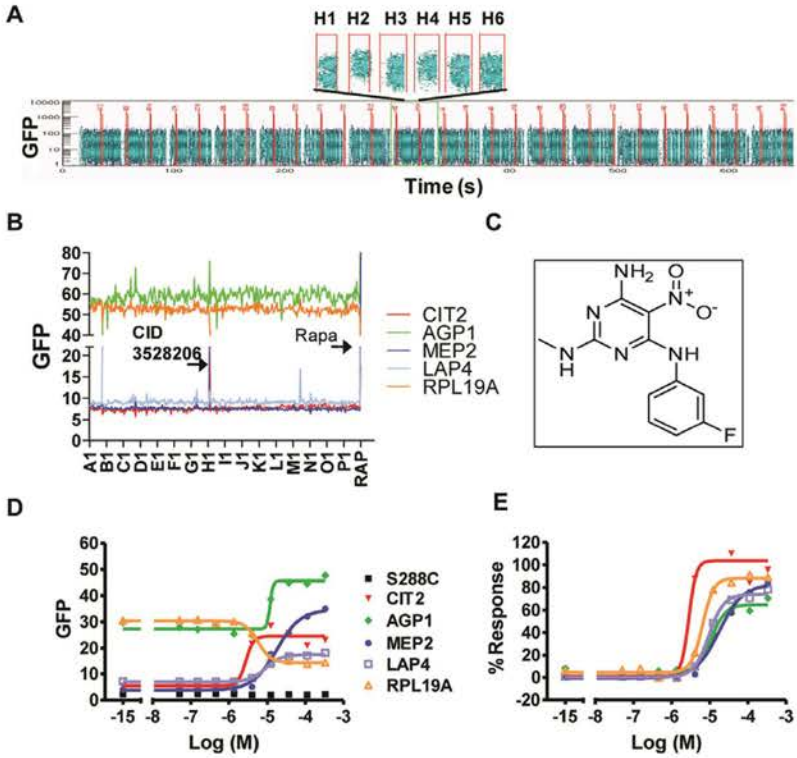


Figure 2. Confirmation of CID 3528206 as a hit from the multiplexed HTS. (A) Screen shot of sample plate ML32337. Each block represents a group of 20 samples and 2 controls (one positive and one negative control), separated by blank wells. Each sample contains data for 5 targets. The negative control (DMSO, well H1), positive control (rapamycin, well H2), and a potential hit (well H4) are shown above. (B) The GFP signal of each strain in each well of plate ML32337 was analyzed by HyperView software. The arrows denote the hit in well H4 (CID 3528206) and the rapamycin positive control. (C) Chemical structure of CID 3528206. (D, E) Dose-dependent regulation of GFP expression in yeast GFP clones by CID 3528206. Raw median channel fluorescence of GFP signal (D) and percent response with respect to rapamycin (E) of each GFP clone are graphed.

with distinct structure as well as molecules selective for individual branches that could target effectors in the TORC1 pathway or interfere with other non-TOR, cross-talk signaling mechanisms. These molecules would represent new chemical tools for delineation of the yeast TOR pathway or serve as potential drug leads for mTOR-based therapies.

Here we report the screening results from the Molecular Libraries Small Molecule Repository (MLSMR) consisting of ~320,000 compounds. We identified a small molecule CID 3528206 that alters GFP expression in all five GFP clones in a manner analogous to that of rapamycin and showed that this molecule behaved in a manner consistent with inhibition of yeast TORC1 in follow-up biochemical and cell-based assays.

RESULTS AND DISCUSSION

Identification of Rapamycin-Responsive Yeast GFP Clones. To determine the appropriate TORC1-regulated GFP clones for HTS, we evaluated the yeast GFP collection for rapamycin-responsive clones. A total of 106 GFP clones were identified as responsive to rapamycin in YPD media from the primary screen, and 58 clones were confirmed (Supplemental Table S1). GFP fluorescence was down-regulated in 18 clones and up-regulated in 40 clones. Note that all down-regulated clones encode ribosomal proteins, which is in agreement with the fact that rapamycin inhibits protein synthesis. Twenty-six of the up-regulated clones encode proteins involved in RTG, NDP, SPS,[36] and stress response pathways that are known to be regulated by the TORC1 pathway (Supplemental Table S1). Other rapamycin up-regulated clones encode proteins that have not yet been linked to the TORC1 pathway, including several uncharacterized ORFs (Supplemental Table S1). Characterization of these GFP clones may identify novel components in the yeast TORC1 pathway and may prove valuable for evaluation of compounds identified from the subsequent HTS. Since YPD medium compromised the multiplexing staining protocol (see below), we retested 96 positive GFP clones from the primary screen in SC media. A total of 50 rapamycin-responsive GFP clones were confirmed in SC media (Supplemental Table S1). To determine the GFP clones for yielding good Z' (>0.5), a statistical factor to assess the quality of HTS,[37] we picked the ones that showed a robust response to rapamycin in SC media for a 3-h time-course study (Figure 1B and data not shown). With DMSO treatment, the GFP signal in the *CIT2*, *AGP1*, *MEP2*, and *LAP4* clones remained constant over time. With rapamycin treatment, the GFP signal in these clones increased greater than 3-fold at 3 h compared to DMSO treatment. In contrast, the GFP signal in the *RPL19A* clone increased steadily with DMSO treatment but remained unchanged with rapamycin treatment, resulting in a 1.5-fold decrease of the GFP signal with rapamycin treatment compared to DMSO treatment. We selected these five GFP clones, *CIT2*, *AGP1*, *MEP2*, *LAP4* and *RPL19A*, representing four signaling

branches of the yeast TORC1 pathway for multiplexed HTS (Figure 1A).

Development of a 5-Plex Strategy. Multiplexing is a powerful capability of flow cytometry and various strategies have been developed for beads as well as both fixed and live cells.[38] We created a live cell-based multiplex using "barcoding" to discriminate the different clones in the multiplex. We used Alexa Fluor 405 (Ex/Em: 400/424 nm) and/or Alexa Fluor 633 (Ex/Em: 632/647 nm) to stain the yeast cells at two levels of fluorescence intensity and then interrogated the cells for changes in GFP expression (Ex/Em: 488/507 nm). The staining scheme is shown in Figure 1C. In Figure 1D (0 h), we can readily distinguish five distinct cell populations (gates 1−5). To test the stability of the barcode, we analyzed the stained multiplex at hourly intervals up to 4 h under normal yeast growth condition (Figure 1D and data not shown). After 3 h incubation, these five cell populations remained distinguishable (Figure 1D, 3 h), and when gating on a discrete population, the resulting GFP histogram was a homogeneous population with negligible contamination from other clones. However, a distinct population (~50%) of new-born daughter cells was generated at 3 h (Figure 1D, 3 h, gate 6), and it dominated the culture (80%) after 4 h (data not shown). As only the stained cells (mother cells) in gates 1−5 are analyzed, too many daughter cells result in insufficient number of mother cells for data analysis. To balance the duration of compound incubation and mother cell number, we selected 3 h for HTS.

Identification of Compounds That Modulate Expression of *GFP* Fusion Genes. HTS was carried out in a 5-plex assay format in which *CIT2*, *AGP1*, *MEP2*, *LAP4*, and *RPL19A* GFP fusion clones were color-coded with Alexa Fluor dyes and evaluated simultaneously with respect to the alteration of GFP expression. Gating based on FL6 and FL8 emission distinguished the five GFP clones as shown in Figure 1D. The original screening data and the intensity of GFP signal of each clone in each well from the sample plate ML32337 are displayed in Figure 2A and B, respectively, showing the hit compound CID 3528206 in well H4. The average Z' were all above 0.5 for each target clone (data not shown). Primary screening of the MLSMR (~320,000 compounds) in 5-plex format resulted in the identification of 210, 51, 1682, 1090, and 982 active compounds for *CIT2*, *AGP1*, *MEP2*, *LAP4*, and *RPL19A* clones, respectively (Table 1). The complete results

Table 1. Hits Summary from the Primary and Confirmatory Screening

targets	*CIT2*	*AGP1*	*MEP2*	*LAP4*	*RPL19A*
primary hits[a]	210	51	1682	1090	982
confirmed hits[b]	19	19	12	205	102

[a]A total of ~320, 000 compounds were screened. [b]A total of ~2,500 compounds were screened in single point confirmation. A total of 613 compounds were screened in dose−response confirmation. A total of 255 hits were confirmed.

from the multiplex screen are available on PubChem (PubChem summary AID 1908, http://pubchem.ncbi.nlm.nih.gov). A total of 255 active compounds were confirmed: 19, 19, 12, 205, and 102 actives for the *CIT2*, *AGP1*, *MEP2*, *LAP4*, and *RPL19A* clones, respectively (Table 1). We then analyzed the distribution of these 255 confirmed compounds among the five GFP clones. A total of 176 compounds selectively targeted one clone, while 79 compounds targeted multiple clones, among which 1, 2, 16, and 60 compounds altered GFP expression in five, four, three, and two clones, respectively (Table 2). This distribution pattern suggests that we may have identified compounds that target either various signaling nodes in the yeast TORC1 pathway or target pathways engaged in cross-talk with TORC1. Notably, a large number of hits (67 in total) target both *LAP4* and *RPL19A* clones. We observed that dozens of *LAP4* hits that were statistically inactive for *RPL19A* in the primary single-point screen were confirmed active for the *RPL19A* clone in the dose−response analysis. This observation may suggest the co-regulation of *LAP4* and *RPL19A* branches.

While all of these molecules are interesting and worth pursuing, subsequent characterization and target identification remains challenging. For instance, the hits for *LAP4-GFP* clone may act on pathways including PKA, autophagy, stress response, and TORC1 pathways. Since it is unlikely that one can design a simple assay to clarify all these hits, we focused on the hit CID 3528206 that most likely acts on TORC1 as it alters GFP expression in all five GFP clones in an analogous manner to rapamycin. We are also characterizing the LAP4 hits to validate potential autophagy regulators (data not shown).

Purified CID 3528206 powder was obtained to confirm its activity in the dose−response assay (Figure 2D and E). CID 3528206 increased GFP expression in the *CIT2*, *AGP1*, *MEP2*, and *LAP4* clones and decreased GFP expression in the *RPL19A* clone (Figure 2D). Its EC_{50} on all clones ranges from 3 to 13 μM. The percentage response of the clones to CID 3528206 varied from 40% to 100% compared to rapamycin (Figure 2E). This indicates that this small molecule represents a distinct chemical structure that may function like rapamycin (Figure 2C).

Compound CID 3528206 Inhibits Yeast Cell Growth but Is Not Toxic. As rapamycin arrests yeast cell growth,[39,40] we first determined the toxicity and effect of CID 3528206 on cell growth. Compared to DMSO treatment, CID 3528206 significantly inhibited cell growth at 20 μM (Figure 3A). The growth inhibition caused by CID 3528206 was dose-dependent (Figure 3B). To test if CID 3528206 is toxic to yeast cells, we evaluated yeast viability following treatment with 30 μM CID 3528206 by a standard colony forming unit (cfu) assay (Figure 3C). Similar to DMSO and rapamycin treatments, greater than 90% of the cells treated with 30 μM CID 3528206 were viable at all three time points analyzed. In contrast, a 4-h treatment with the fungicidal compound amphotericin B reduced the corresponding cfu to levels below 0.5% when compared to control cells. These data indicate that CID 3528206 inhibits yeast cell growth but is not toxic at a concentration of 30 μM.

CID 3528206 Inhibits Yeast Cell Growth through Modulation of TORC1. Rapamycin inhibits TORC1 activity allosterically when complexed with Fpr1p and thus exhibits a cytostatic effect on wild-type yeast cell growth. When the downstream TORC1 effectors are constitutively active, cells can bypass TORC1 activity and resist rapamycin treatment, such as with the TORC1 bypass cells bearing $SCH9^{2D3E}$ and *GLN3* KO alleles.[41,42] We tested whether the TORC1 bypass cells resist CID 3528206 treatment (Figure 3D). Consistent with the previous report, rapamycin inhibited wild-type cell growth but not TORC1 bypass cell growth at 0.2 μM.[41,42] CID 3528206 also inhibited wild-type cell growth but not TORC1 bypass cell growth at 30 μM, indicating that the growth inhibition effect of CID 3528206 is mediated by TORC1, and suggesting that CID

Table 2. Distribution of Confirmed Hits[a]

	no. of hits															
	1	2	1	4	1	4	6	5	3	2	50	6	7	2	128	33
CIT2	*	*	*	*				*				*				
AGP1	*		*		*	*			*	*			*			
MEP2	*	*			*		*							*		
LAP4	*	*	*	*	*	*	*	*	*		*				*	
RPL19A	*	*		*		*	*			*	*					*

[a]Asterisk (*) indicates active for the indicated GFP clones.

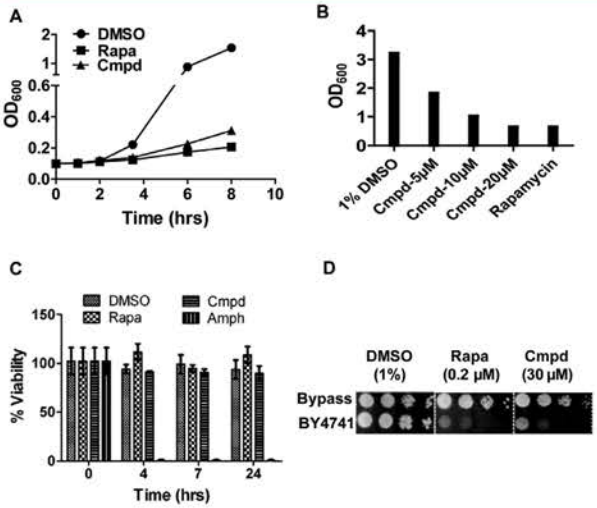


Figure 3. CID 3528206 inhibits yeast cell growth but is not toxic. (A) CID 3528206 inhibits yeast cell growth. BY4741 cells were treated with 1% DMSO, 0.22 μM rapamycin, and 20 μM CID 3528206 (Cmpd) in duplicate, respectively. OD_{600} was measured at different time points. (B) Dose-dependent inhibition of yeast cell growth by CID 3528206. BY4741 cells were treated with varying concentrations of CID 3528206 (Cmpd) or control compounds in duplicate. OD_{600} was measured after 5.5 h treatment. (C) Yeast viability assay. BY4741 Cells were treated with 30 μM CID 3528206 (Cmpd), rapamycin (Rapa), amphotericin B (Amph), and DMSO in triplicate, respectively. OD_{600} and cfu were measured at different time points, and percent viability was calculated by cfu's relative to the number of cells. (D) CID 3528206 inhibited growth of wild-type cells but not the TORC1 bypass cells. A series of 10-fold dilutions of BY4741 and TORC1 bypass cells were spotted onto YPD plates containing indicated concentrations of CID 3528206 or control compounds and grown for 3 days.

3528206 either acts, like rapamycin, directly on TORC1, or upstream of TORC1.

CID 3528206 Inhibits TORC1 *in vivo* and *in vitro*. To investigate whether CID 3528206 inhibits TORC1, we first examined the *in vivo* phosphorylation of Sch9p (a direct substrate of TORC1).[42] Like rapamycin, CID 3528206 caused dose-dependent dephosphorylation of the C-terminal phosphorylation sites in Sch9p (Figure 4A). TORC1 activity was quantified in Figure 4B, and IC_{50} was calculated as 3.9 μM, which is consistent with the range of EC_{50}'s of all clones shown in Figure 2D. This result indicates that CID 3528206 inhibits TORC1 activity *in vivo*, suggesting that CID 3528206 acts on TORC1 or upstream. To ask if CID 3528206 targets TORC1 directly, we performed TORC1 *in vitro* kinase assay (Figure 4C and D). CID 3528206 completely inhibited Sch9p phosphorylation at 1 μM or higher concentrations. CID 3528206 also inhibited Kog1p autophosphorylation to the greatest extent (~70% inhibition) at 1 μM or higher concentrations. Dose–response curve-fitting showed that CID 3528206 inhibited both Sch9p and Kog1 phosphorylation with IC_{50}'s of ~150 nM (Figure 4D). This result favors the possibility that CID 3528206 targets TORC1 directly rather than acting on its upstream components. Further identification of TORC1 mutants resistant to CID 3528206 could confirm this hypothesis. Note that the *in vitro* IC_{50} (150 nM) is 20-fold lower than the *in vivo* IC_{50} (3.9 μM), which may be due to cell permeability or efflux systems that result in a lower cellular concentration of CID 3528206.

Genome-Wide Comparison of CID 3528206 with Rapamycin on Yeast Gene Expression. To compare the effect of CID 3528206 and rapamycin on yeast gene expression, we performed microarray analysis using Affymetrix yeast gene chips (Figure 5A). CID 3528206 significantly altered the transcription of 751 yeast genes, among which 477 genes were up-regulated and 274 genes were down-regulated. Rapamycin significantly increased the transcription of 512 genes and decreased the transcription of 257 genes. Venn diagram analysis showed that 422 genes up-regulated by CID 3528206 (88.5%) were also up-regulated by rapamycin, while 217 genes that were down-regulated by CID 3528206 (79.2%) were also down-regulated by rapamycin. Overall, CID 3528206 and rapamycin shared 85.1% regulated genes. Such a significant overlap in gene expression profiling indicates that CID 3528206 and rapamycin modulate similar cellular pathways. Since the mRNA levels do not necessarily reflect the protein levels, we took advantage of the rapamycin-responsive GFP clones identified in the yeast GFP collection to test whether CID 3528206 altered GFP expression in these clones (Figure 5B). The GFP signal in 14 out of 17 rapamycin-down-regulated clones was decreased by CID 3528206. Note that GFP expression in the other three rapamycin-down-regulated clones was also decreased by CID 3528206 to 0.67–0.72-fold, which is slightly higher than the 0.66-fold cutoff value. This result indicates that CID 3528206 down-regulated GFP expression in almost all rapamycin down-regulated clones. The GFP signal in 19 out of 33 (57.6%) rapamycin-up-regulated clones was up-regulated by CID 3528206. Overall, CID 3528206 altered GFP expression in 33 out of 50 (66.0%) rapamycin-responsive GFP clones. This significant overlap is in good agreement with the microarray data and confirms the common cellular function of CID 3528206 and rapamycin.

Preliminary Structure–Activity Relationship (SAR) Exploration. To explore the SAR optimization, we surveyed 57 analogues where the substitutions were focused on four regions of the scaffold (Figure 6A, shaded regions). Alteration of the free primary amino substituent (Figure 6A, green shaded region) or modification of the nitro group (Figure 6A, blue shaded region) was not tolerated. Modest changes in alkyl

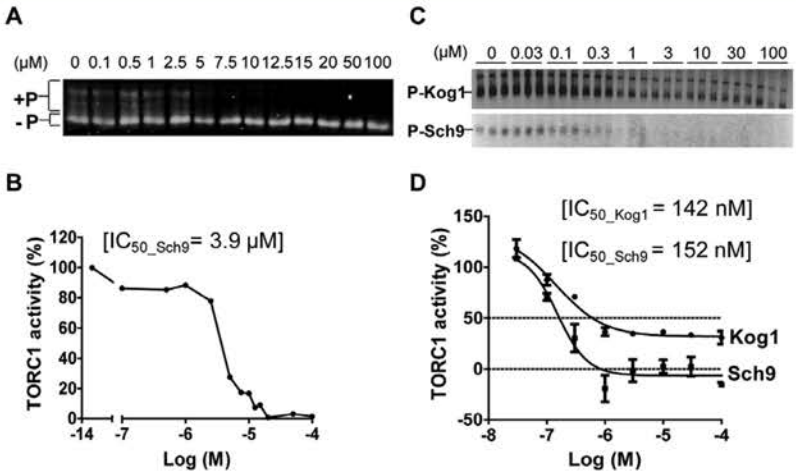


Figure 4. CID 3528206 directly inhibits TORC1. (A, B) CID 3528206 inhibited TORC1 activity *in vivo*. Yeast cells expressing Sch9p-5HA were treated with DMSO vehicle or varying concentrations of CID 3528206 for 30 min. (A) Western blots using anti-HA antibody to detect the extent of Sch9p C-terminal phosphorylation;. (B) The ratio of the hyperphosphorylated over the hypophosphorylated Sch9p was used to quantify TORC1 activity *in vivo*. (C, D) CID 3528206 inhibited TORC1 activity *in vitro*. *In vitro* kinase assay using Sch9p as substrate was performed in triplicate (C) and quantified as mean ± SD (D).

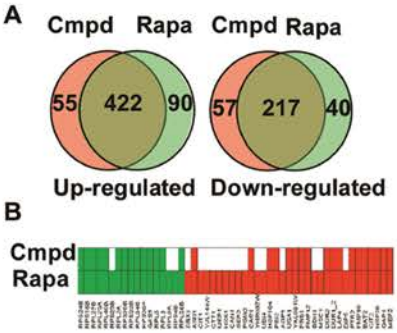


Figure 5. CID 3528206 shares common cellular function with rapamycin. (A) Venn diagram analysis of the CID 3528206-regulated genes and rapamycin-regulated genes identified from microarray analysis. (B) CID 3528206 altered GFP expression of rapamycin-responsive GFP clones. Fifty rapamycin-responsive GFP clones (Supplemental Table S1) were grown in SC media and treated with DMSO, 30 μM CID 3528206, or 0.22 μM rapamycin respectively for 3 h in triplicate. GFP fluorescence was measured by flow cytometry. A 1.5-fold change in fluorescence was used as the cutoff value for up-regulated (red) and down-regulated (green) clones.

substituent of the methyl amine (Figure 6A, yellow shaded area) led to one active compound, but with an altered activity profile (data not shown). Interestingly, changes to the 3-fluorophenyl appendage (Figure 6A, purple shaded region) afforded a set of analogues that appeared to exclusively alter CIT2 GFP expression, unlike CID 3528206, which modulated GFP expression in all five GFP clones (Figure 6B). Surprisingly, these CIT2 branch-selective analogues also inhibited Sch9p phosphorylation *in vivo* (Figure 6B). Due to solubility challenges with some analogues in this series (data not shown), the possible link between physiochemical properties and CIT2 branch selectivity is being investigated with the pursuit of compounds with enhanced solubility.

Conclusion. In summary, the flow-cytometry-based multiplexing HTS has successfully identified a yeast TORC1 inhibitor and provided a novel scaffold for further development of TOR inhibitors. Interestingly, preliminary SAR exploration on the single chemotype identified analogues exhibiting both rapamycin-like activity and *CIT2/RTG* branch selective activity. Further investigation of structural and biological basis of the analogues may reveal novel mechanisms in the yeast TORC1 pathway. As the TOR complexes and signaling pathways are highly conserved,[2,3] CID 3528206 may have potential for inhibiting mTORC1 activity. Characterization of the activity of CID 3528206 in other organisms and comprehensive SAR analysis are under way to elucidate the mechanism of action of CID 3528206.

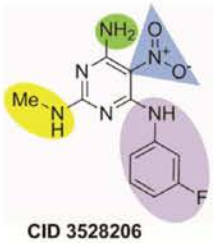
METHODS

Yeast Strains and Reagents. Yeast strains and plasmids used in this study are listed in Supplemental Tables S2 and S3, respectively. The yeast GFP collection (Invitrogen) was screened as described previously.[43]

The fluorescent dyes Alexa Fluor 405 and Alexa Fluor 633 as well as Pluronic F-68 were purchased from Invitrogen. Rapamycin was purchased from TOCRIS Biosciences.

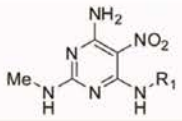
Multiplexed HTS. The *CIT2*, *AGP1*, *MEP2*, *LAP4* ,and *RPL19A* GFP fusion clones were grown separately overnight in 15 mL of SC media at 30 °C to 0.5−1.0 OD_{600}. Approximately 10 OD_{600} cells were washed with PBS buffer once and resuspended in 10 mL of PBS buffer supplemented with 0.03% (v/v) Pluronic F-68. Different combinations of Alexa Fluor 405 (0, 16, 100 μL of 1 mg mL^{-1} DMSO stock solution) and Alexa Fluor 633 (0, 4 μL, 100 μL of 1 mg mL^{-1} DMSO stock solution) dyes were added to cells and incubated for 45 min at RT. Cells were washed with PBS twice and resuspended in SC media. Stained cells were combined and diluted into fresh SC media at 0.5 OD_{600}. Ten microliters of mixture was added to each well in 384-well plates in the sequence below: first, 5 μL of SC media supplemented with 0.03% Pluronic F-68; second, 100 nL of compounds; third, 5 μL mixed cells. Plates were incubated at 30 °C upside down for 3 h. The cells in the multiplex were interrogated for GFP expression levels using the established HTFC at UNMCMD.[44] Flow cytometric light scatter and fluorescence emission at 530 ± 20 nm (FL1), 665 ± 10 nm (FL8) and 450 ± 25 nm (FL6) were collected. The resulting time-gated data files were analyzed with HyperView software to determine compound activity in each well. A compound was considered active if the change in fluorescence was greater than 50% of DMSO-treated controls. The quality control statistic Z'[37] for each GFP clone was calculated with the

A



CID 3528206

B



Entry	CID	R1	EC50 (μM) [a](% Response) CIT2	AGP1	MEP2	LAP4	RPL19A	[b]EC50_Sch9 (μM)
1	3528206	3-F-phenyl	3.4 (94.7%)	11.3 (39.2%)	12.7 (77.2%)	9.1 (59.1%)	6.4 (69.9%)	3.9
2	3392161	3-Cl-phenyl	3.8 (91.7%)	- (5.5%)	- (16.2%)	- (9.1%)	- (16.4%)	2.3
3	3449329	phenyl	8.8 (97.7%)	- (10.5%)	- (5.5%)	- (8.2%)	- (20%)	14.7
4	4359574	2-MeO-phenyl	4.0 (32.6%)	- (15.4%)	- (3.3%)	- (4.4%)	- (7.5%)	5.9
5	3739601	4-F-phenyl	56.1 (76.2%)	- (17.1%)	- (0.4%)	- (5.1%)	- (20%)	N/D
6	4293401	2-Br-phenyl	3.8 (29.7%)	- (17.2%)	- (3.8%)	- (7.7%)	- (20%)	N/D
7	50904400	3-Br-phenyl	0.6 (22.6%)	- (9.1%)	- (6.4%)	- (3%)	- (9.3%)	N/D
8	50904405	3-MeO-phenyl	3.9 (29.8%)	- (3.5%)	- (7.0%)	- (5.5%)	- (15.8%)	N/D

[a]Percent response with 100 μM test compound; [b]EC_{50} of Sch9p phosphorylation assay described in Figure 4A; -: inactive; N/D: not determined

Figure 6. SAR optimization of CID 3528206. (A) Shaded regions of targeted SAR optimization. (B) Active analogues generated from changes to the 3-fluorophenyl appendage (purple shaded region). The results of dose–response and *in vivo* Sch9p phosphorylation assays are shown.

GFP fluorescence of rapamycin- and DMSO-treated controls. Active compounds were further confirmed in single point and dose–response analyses in a single-plex format. A counter-screen for green fluorescent compounds was also performed using a non-GFP parental strain S288c.

Yeast Viability and Cell Growth Assays. Colony forming unit (cfu) assay was performed to evaluate yeast viability. BY4741 cells were treated with 1% DMSO, 0.22 μM rapamycin, 30 μM CID 3528206, or 2.5 μg mL^{-1} amphotericin B in triplicate for 24 h. OD_{600} and cfu's were measured at 4, 7, and 24 h. Percent viability was calculated by cfu's relative to the number of cells in the culture. The TORC1 bypass (MP138-4C cell containing p1290) and BY4741 cells spot assay was performed as described previously.[41,42]

Sch9p *in vitro* and *in vivo* Phosphorylation Assays. Sch9p *in vitro* phosphorylation assay was performed as described previously.[41,42] Sch9p *in vivo* phosphorylation assay was performed using YL515 cells containing plasmids pRS413, pRS416, and pJU1058 in the chemical fragmentation analysis as described.[41,42]

Microarray Analysis. Overnight cultures of BY4741 cells in YPD were diluted to 0.05 OD_{600} and allowed to grow to 0.2 OD_{600}. Cells were treated with 0.22 μM rapamycin, 20 μM CID 3528206, and 1% DMSO vehicle control in duplicate for 3 h, respectively. Cells were collected, and RNA was isolated with MasterPure Yeast RNA purification kit (Epicentre). cDNA probes were generated from total RNA and used for hybridization to yeast gene chips (Affymetrix) at Keck-UNM Genomics Resource. The RMA algorithm of Expression Consol (v1.1, Affymetrix) was used to generate and normalize signal intensities. The significant analysis of microarray (SAM)[45] was performed with a false discovery rate (FDR) of 0.1 to identify the genes whose expressions are significantly up- or down-regulated by CID 3528206 or rapamycin compared to DMSO treatment. The microarray data (Accession number GSE33320) are available at http://www.ncbi.nlm.nih.gov/geo.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

We thank S. Chavez, D. Perez, A. Evangelisti, M. Garcia, V. Salas, K. Gouveia, G. Pickett, E. Manzanilla, K. Niewola-Staszkowska, and S. Haesendonckx for technical assistance. This research was supported by National Institutes of Health grants U54MH084690, U54HG005031 and R03 MH086450-01 and a grant from the Swiss National Foundation (CDV).

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Aknowledgments

I am grateful to Professor Claudio de Virgilio for taking me into his laboratory and for transmitting me his enthusiasm for research. I really appreciated that he always found the time to discuss with me and that he was so involved in the projects. He asked me a lot and challenged me, but in return he gave me a lot and led me to extract the best of myself.

I would like to thanks Professor Aurelio Teleman and Andreas Mayer for accepting to review this thesis and being experts in the jury of my PhD defense.

This work won't have been possible without the support of my fantastic colleagues. Many thanks to Marie-Pierre who taught me so many technics and tricks, who transmitted me her systematic and efficient way of working, and who always had a nice word to motivate me even in the hardest moment. Thank you to Floriane for everything she does for the laboratory and for me. Everything she touches is becoming gold. I would also like to thank Katie for the comments on this manuscript, Malika for the technical support, and Jean-Daniel and Olga for rendering our every-day job easier. Thank you to Marta, Serena, Alessandro, Emma, Nicolas, Xuan, Grégory and Matteo for all the nice time we shared inside and outside the lab.
I also would like to thank people from the neighboring laboratories, especially Professor Dieter Kressler for his advices and for the interesting discussions we had. And also Shirish, Fernando, Rabih, Christian, Nicolas Jacquier, Fred, Sylvie, Dagmar, Guillaume, Hector and Arlette.

Thank you to my friends who helped me keeping the balance through cycling, music or just sharing nice moments: Gregoire, Fabian, Xavier, Tritri, Thierry, Bobich, David, Jacky, Sciboz, Daniel, Jb and the FREI! crew.

I am grateful to my parents, grandparents and sister who stimulated my curiosity from day 1, who always supported me, and gave me the opportunity to study.

Finally there is no word to express my gratitude and my love to my wife Marlene, who renders every day more beautiful than the previous one since I know her.

Curriculum Vitae

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Experience:

Research Assistant, University of Fribourg
January 2010 – Present

- Purification of recombinant proteins (affinity and size exclusion chromatography)
- Tandem-affinity protein purification from yeast.
- Protein analytics and characterization (phosphorylation, localization, interactions).
- Cloning (PCR, plasmid construction)
- Confirmed results of High-Throughput screens for small-molecules drugs.
- Gave practical courses and supervised students in small groups and large classes.
- Scientific writing, literature search, data management and presentation.

Developed expertise in Biochemistry and in Molecular and Cell Biology. Learned to work independently, to manage my time and to solve problems by myself. Managed and scheduled the work of a technician. Developed teaching and leading skills.

Research Assistant, Swiss Tropical and Public Health Institute (Zivildienst)
March 2009 – October 2009 (8 months)

-Worked at the Central Veterinary Laboratory of Bamako, Mali, and managed a field-work project on Brucellosis.
Organisation of data collection in difficult conditions. Worked in a culturally different environment.

Socio-cultural Worker Assistant, Rest-Home "La Sombaille",la Chaux-de-Fonds (Zivildienst) January 2004 – May 2004 (5 months)

- Assist people through different social activities.

Developed listening and communication skills.

Various student jobs (1998-2009)
Butcher, Driver for directors and actors at Fribourg International Film Festival, Mover,…

Education :

PhD, Biochemistry, University of Fribourg
2010-Present
Thesis on nutrient sensing in budding yeast under supervision of Prof. Claudio de Virgilio.

Master of Science (M. Sc.), Biochemistry, University of Fribourg
2007-2009
Master work on protein-protein interactions in the laboratory of Prof. Claudio de Virgilio.
Introduced a new technique in the lab. Supervised two bachelor students.

Bachelor of Science (B. Sc.), Biology, University of Fribourg
2004-2007
Bilingual bachelor German/French. Worked in the laboratory of Dr. Liliane Sticher on *A.thaliana*.

Technical Skills :

-**Molecular Biology**: PCR, qRT-PCR, RNA extraction, DNA gel electrophoresis, cloning, gene deletion and genomic tagging in yeast, site-directed mutagenesis.

-**Cell Biology**: protein extraction, SDS-PAGE, Western Blot, protein phosphorylation analysis, fluorescence microscopy, protein-protein interactions , split-ubiquitin-based yeast two hybrid system.

-**Biochemistry**: recombinant protein purification from bacteria, Äkta Prime Plus FPLC system, size exclusion chromatography, tandem-affinity protein purification from yeast, *in vitro* protein interaction, *in vitro* GTP hydrolysis assays.

IT skills : MS Office, basics in Adobe Photoshop and Illustrator, basics in bioinformatics tools (blast, multiple alignments, motifs scan, Expasy tools), Serial Cloner, basic statistics on Prism (t-test, ANOVA, regressions).

Languages :

French	:	Native proficiency.
English :		Full professional proficiency.
German	:	Professional working proficiency.
Spanish	:	Good understanding, basic conversation.

Honors and Awards :

Thürler-Reeb Prize 2014 for excellence of the publication “Amino Acid Deprivation Inhibits TORC1 Through a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr1”, Science Signaling, 2013.

Extracurricular activities :

-Co-founder and committee member of FREI!, a non-profit association which organized more than forty concerts since 2002 and promoted local bands.

-Former president (2008) and committee member (2006-2007) of the Ecuvillens-Posieux village association “Union” which organises different cultural events such as theatre and the Fribourg traditional “Bénichon”.

Learned to motivate volunteers, to take responsibilities and to manage a budget.

Interests :

Innovation, Live music, road cycling, martial arts, playing guitar.
Practice of music instruments and martial arts taught me to be hard working and persevering.

References :

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Publications :

SEACing the GAP that nEGOCiates TORC1 activation: Evolutionary conservation of Rag GTPase regulation. (2013)
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Amino Acid Deprivation Inhibits TORC1 Through a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr1. (2013)
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Identification of a Small Molecule Yeast TORC1 Inhibitor with a Multiplex Screen Based on Flow Cytometry. (2012)
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Eukaryotic Cell. 10(10):1367-9

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

Meetings and Conferences :

June 2013:	71st SGM-SSM Annual Meeting, Interlaken, Switzerland. Poster presentation.
March 2013:	Talks about TORCs: recent advances in target of rapamycin signalling. Biochemical Society, London, UK. Poster presentation.
September 2011:	TOR, PI3K and AKT : 20 years on. Basel, Switzerland.
September 2010:	Swiss yeast meeting, Fribourg, Switzerland.
June 2009:	Laboratoire Central Vétérinaire, Bamako, Mali. Invited speaker.