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The EGO complex controls TORC1 in response to amino acids

THESE

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2. Abstract

Amino acids are key building blocks for protein synthesis and many metabolic processes. Amino acids are also key signalling molecules that impinge on signalling pathways that control synthesis and degradation of proteins, hence are central in the process of growth control. The *S. cerevisiae* *t*arget *o*f *r*apamycin *c*omplex *1* (TORC1) is a large protein complex essential for growth that controls protein synthesis, cell growth and mass accumulation in response to glucose and to the quantity and quality of nitrogen. The yeast readouts of TORC1 are quite well understood and characterized, but so far, little is known on the mechanisms that control TORC1. This work identifies the EGO Complex (EGOC), which is composed of Ego1, Ego3, Gtr1 and Gtr2, and which is essential for *e*xit from rapamycin-induced *gro*wth arrest, as an activator of TORC1 in response to amino acids. Moreover, it will be shown that the nucleotide binding status of the EGOC subunit Gtr1 is regulated by the GEF Vam6 and is critical for TORC1 activity control. The data further indicate that the nucleotide binding status of Gtr1 is maybe controlled by the level of intracellular amino acids. Finally, it will be discussed the possibility that both cytoplasmic and vacuolar amino acid pools may contribute to the activation of TORC1.

3. Summary

In nutrient sensing, signal transduction refers to any molecular process that couples availability of nutrients with cell growth. To support growth, nutrients must be sensed in the environment, transported inside the cells, metabolized and eventually used for protein synthesis and mass accumulation.

The *m*ammalian *t*arget *o*f *r*apamycin *c*omplex *1* (mTORC1) is a protein complex that is controlled by nutrient availability, and that controls important aspects of growth like mass accumulation, cell growth and protein synthesis. Budding yeast *Saccharomyces cerevisiae* is a small eukaryote used in many laboratories as model organism for higher eukaryotes (*e.g.* mammals, fruit flies, nematodes). Yeast, like mammalian cells, contain a TORC1 complex composed by the serine/threonine kinases Tor1 and/or Tor2 (mTOR), Kog1 (raptor), Lst8 (mLst8) and Tco89 (which occurs only in yeast). Many readouts of TORC1 have been identified and are well understood. In general, TORC1 promotes protein synthesis and other anabolic processes, while it inhibits macroautophagy and other catabolic and stress-response processes. However, it is not clear what controls TORC1 in yeast. Many studies pinpointed glutamine as key regulator of TORC1, but also glucose was indicated as key metabolite for activation of this complex. Recent findings identified the EGO Complex (EGOC) essential for *e*xit from rapamycin-induced *gro*wth arrest as a possible activator of TORC1. The EGOC is located at the vacuolar membrane and it is composed of Ego1, Ego3 and the two Rag GTPases Gtr1 and Gtr2, yeast hortologues of the mammalian RagA/B and RagC/D respectively. In mammals, Rag GTPases have been shown to bind and activate mTORC1 in response to amino acids by relocalizing mTORC1 to a specific intracellular compartment where it is activated by the GTP-binding protein Rheb.

In this study it will be shown that the yeast EGOC is essential to mediate an amino acid signal to TORC1. In particular, the RagA/B hortologue Gtr1 binds directly to TORC1 (presumably via the conserved subunit Kog1) in a leucine-dependent manner. In cells lacking EGOC subunits, TORC1 displays reduced activity and cells are hypersensitive to rapamycin and fail to recover after a rapamycin-induced growth arrest. The nucleotide loading status of Gtr1 is crucial for its activity: an allele of Gtr1 that mimics the GTP-bound conformation stays bound to TORC1 even in the absence of leucine and partially prevents TORC1 inactivation following leucine deprivation. In addition to these data, the Vam6 GEF, already know for its role in vacuolar fusion events, is proposed to function as a the *g*uanine nucleotide *e*xchange *f*actor for Gtr1. Both genetic and biochemical

evidences are favouring a model by which Vam6 has a dual role in controlling vacuolar fusion by acting on Ypt7 and controlling TORC1 activity by stimulating the GDP for GTP exchange on Gtr1. However, it is not clear if Vam6 can be also a sensor for amino acid availability and it is possible that other regulatory pathways may act in parallel to Vam6 in controlling TORC1 via the EGOC. Interestingly, unlike in mammalian cells, leucine deprivation does not seem to affect the vacuolar localization of TORC1 in yeast, indicating that some aspects of EGOC-mediated control of TORC1 may not be conserved.

How the EGOC senses amino acids is not clear. Addition of the inhibitor of translational elongation cycloheximide (CHX) to cycling cells triggers a quick and massive hyperactivation of TORC1 probably because it results in a robust increase in the intracellular pools of amino acids. In addition, TORC1 is still active in prototrophic strains grown on media lacking amino acids indicating that the stimulus sensed by TORC1 is intracellular rather than environmental. In particular, it must be stated that amino acids are stored in the yeast vacuole and used in the cytoplasm for protein synthesis. Thus, it is extremely important for cells to continuously monitor both pools for proper coordination of protein synthesis and cells growth. The data presented suggest that TORC1 may be activated by both cytoplasmic and vacuolar pools with the EGOC acting more likely a sensor of the cytoplasmic pool.

4. Riassunto

Nell'ambito dei processi di captazione dei nutrienti, con il termine "trasduzione del segnale" si indicano tutti quei processi molecolari che sincronizzano la disponibilità dei nutrienti con la crescita cellulare. Prima di poter essere utilizzati per la crescita, i nutrienti devono essere captati nell'ambiente di crescita, trasportati all'interno delle cellule, metabolizzati ed infine utilizzati per la sintesi proteica e per la creazione di nuova massa cellulare.

Il complesso mTORC1 di mammifero (*m*ammalian *t*arget *o*f *r*apamycin *c*omplex *1*) è un complesso proteico che sembra essere controllato dai nutrienti e a sua volta controlla diversi aspetti della crescita cellulare come ad esempio la creazione di nuova massa, la crescita e la sintesi proteica. Il lievito *Saccharomyces cerevisiae* è un piccolo organismo eucariotico usato in differenti laboratori come organismo modello per l'enorme numero di similitudini che ha con gli eucarioti superiori (ad esempio mammiferi, moscerini della frutta e nematodi). Le cellule di lievito, come quelle di mammifero, contengono un complesso TORC1 composto dalle treonine chinasi Tor1 e/o Tor2, Kog1, Lst8 e Tco89 (che peró è unicamente in lievito). Molti read-out di TORC1 sono stati individuati e caratterizzati. Riassumendo, TORC1 promuove la sintesi proteica e altri processi anabolici e, al contrario, inibisce l'autofagia, i processi catabolici in generale e i processi legati alla risposta agli stress. Purtroppo peró, non sono chiari i meccanismi che controllano TORC1 in lievito. Molti studi hanno indicato che la glutammina è un metabolite chiave nella regolazione del TORC1, ma anche il glucosio riveste una propria importanza. Delle recenti scoperte hanno identificato il complesso EGO (*e*xit from rapamycin-induced *gro*wth arrest) come un possibile attivatore di TORC1. EGOC è un complesso proteico localizzato sulla membrana vacuolare e composto dalle proteine Ego1, Ego3 e dalle due Rag GTPasi Gtr1 e Gtr2, ortologhe rispettivamente delle Rag di mammifero RagA/B e RagC/D. In mammifero queste GTPasi interagiscono con il mTORC1 e lo attivano in risposta alla presenza di amino acidi relocalizzandolo in uno specifico compartimento intracellulare in modo da essere attivato da Rheb, suo attivatore già conosciuto.

In questo studio verrà mostrato che l'EGOC di lievito è essenziale per mediare il signale di presenza degli amino acidi al TORC1. In particolare, Gtr1, l'ortologo delle RagA e RagB, si lega direttamente al TORC1 (probabilmente via la subunità Kog1) quando la leucina è presente. In cellule sprovviste di una qualsiasi subunità del complesso EGO, il TORC1 è meno attivo, le cellule sono ipersensibili alla rapamicina e non riescono a sopravvivere ad un blocco della crescita causato da una breve esposizione alla rapamicina. L'attività di Gtr1 dipende dal nucleotide (trifosfato, GTP

o difosfato, GDP) con cui è caricato. Un allele di Gtr1 che mimica la conformazione di Gtr1 caricato con del GTP resta costitutivamente associato al TORC1 anche in assenza di leucina e protegge parzialmente le cellule dagli effetti causati dalla rimozione della leucina. Oltre a questi dati, la proteina Vam6, già conosciuta per il suo ruolo di regolazione della fusione dei vacuoli, è proposta come GEF (*g*uanine nucleotide *e*xchange *f*actor) per Gtr1. Evidenze genetiche e biochimiche favoriscono un modello per cui Vam6 ha un duplice ruolo sia nella regolazione della fusione vacuolare (attivando la GTPase Ypt7) e nel controllo dell'attività del TORC1 stimolando lo scambio GDP/GTP su Gtr1. Non è chiaro peró se Vam6 agisce anche come sensore della disponibilità di amino acidi, ma probabilmente altri pathway agiscono in parallelo a Vam6 nel controllo di TORC1 tramite l'EGOC. In lievito peró la rimozione di leucina non sembra avere un effetto sulla localizzazione del TORC1 indicando che probailmente questo meccanismo non si è conservato durante l'evoluzione.

Come l'EGOC capta gli amino acidi non è chiaro. L'aggiunta dell'inibitore della sintesi proteica cicloeximide (CHX) a cellule in fasi di crescita causa una massiccia iperattivazione di TORC1 probabilmente perchè causa un robusto aumento degli amino acidi intracellulari. L'attività del TORC1 non diminuisce in ceppi prototrofi che crescono in terreni senza amino acidi indicando che lo stimolo che attiva il TORC1 proviene dall'interno della cellula piuttosto che dall'esterno. In particolare bisogna ribadire che gli amino acidi sono conservati nel vacuolo di lievito per essere usati nel citoplasma per la sintesi proteica. É quindi estremamente importante che le cellule continuino a monitorare entrambi i pool amino acidici per coordinare al meglio la sintesi proteica e la crescita cellulare. In questo contesto il TORC1 sembra rispondere sia agli amino acidi citosolici sia a quelli vacuolari e l'EGOC agisce per lo piú come sensore per gli amino acidi citoplasmatici.

5. Literature Overview

5.1. Nutrient sensing and signalling pathways

All living organisms require nutrients that provide the essential building blocks and energy supply to make the necessary cellular components (DNA, proteins, lipids etc.) and drive metabolism. Therefore, the presence of nutrients is essential to survive, develop, and reproduce. As a result, cells have developed sophisticated mechanisms to sense nutrient availability and transduce the corresponding signals via signalling pathways to induce appropriate transcriptional and metabolic responses. Generally, nutrient sensing occur through detection of the nutrient via receptor or transceptor proteins (Kraakman et al., 1999). Alternatively, the presence of certain nutrients can be detected indirectly following their metabolism (Colombo et al., 2004). It is important to state that not only extracellular, but also intracellular nutrient pools are critical for proliferation. Nutrient-induced signalling pathways guarantee optimal nutrient consumption in a dynamic manner and, particularly in unicellular organisms, enable coordinated induction of a resting phase (quiescence)f where cells cease proliferation upon nutrient limitation.

The unicellular eukaryote *Saccharomyces cerevisiae* is often used as a model system to study various aspects of nutrient signalling, proliferation and quiescence. Firstly, because it is easily tractable to all levels of experimental analyses and secondly, because of the conservation of basic cellular processes among eukaryotes (*e.g.* the study of quiescence in yeast is likely to elucidate equivalent mechanisms and states in many other eukaryotes).

In yeast, information about the nutritional state of the environment has to be transmitted across the plasma membrane towards the nucleus, where the genes necessary to survive in specific conditions get activated and are transcribed. Any cellular process necessary to convert an intracellular or extracellular stimulus into a variation in the transcription profile is normally coined as "signal transduction". During signal transduction many different kinds of enzymes are engaged. In the case of fast reactions the environmental signal reaches the nucleus in a matter of few seconds. Conversely, some signalling pathways, like for instance the induction of genes necessary for the metabolization of alternative carbone sources in response to galactose (see below), may take rather minutes to hours to be completed.

When a signal originates in the extracellular environment, the starting point of the transduction pathway is the binding of a certain ligand to a plasma membrane-localized receptor. The receptor is thus often the first protein in the signalling pathway responsible for transmitting the signal across

the membrane into the intracellular environment. The number of proteins involved in the downstream steps of the pathway increases as the process evolves giving rise to a so called "signal cascades". Sometimes second messengers (like cAMP or Ca^{2+}) are part of the signal cascade because of their ability to be rapidly produced and diffused in the cytoplasm. A common feature of all signalling pathways is activation/inactivation of proteins by phosphorylation events triggered by protein kinases. Protein kinases use ATP to transfer a phosphate on a hydroxyl group of a threonine, serine or tyrosine of their target protein (Hanks, 2003; Hanks and Hunter, 1995). Phosphorylation usually results in a conformational modification of the target protein with consequent change of its activity, or localization, or ability to bind other proteins. The activity of the protein kinases is counterbalanced by the action of antagonistic protein phosphatases which are able to remove phosphate groups from phosphorylated proteins.

Other very important proteins involved in signal transduction that are worth mentioning in this work are GTPases. GTPases belongs to a large family of hydrolytic enzymes that can bind and hydrolyze guanosine-triphosphate (GTP) to guanosine-diphosphate (GDP). Their structure is conserved and they play roles in many cellular aspects including signal transduction, protein synthesis, vesicular transport and differentiation. Given the fact that they cycle quickly from an active to an inactive state, they are considered to be efficient molecular switches. Initially, the GTPase binds GDP and is inactive. Upon specific stimulations, GTPases are helped by particular guanosine-nucleotide *e*xchange *f*actors (GEF) to release the GDP and exchange it for GTP (normally more abundant in cycling cells). The GTP-bound GTPase becomes active and able to act on its downstream targets. At this stage, the activity is controlled by the intrinsic hydrolyzing activity of the GTPase that converts the GTP into GDP thereby shutting off the signal. Very often, GTP hydrolysis is stimulated by other regulatory proteins called *G*TPase *a*ctivating *p*roteins (GAPs) (Fig.1). There are other regulators in the GTPase cycle: the *G*TP *d*issociation *i*nhibitors (GDI). This protein associates with the GDP-loaded GTPase and inhibits the release of the GDP molecule thereby maintaining the GTPase in an inactive form (Koch et al., 1997).

All GTPases can be grouped into two distinct major classes: heterotrimeric G-proteins and monomeric small GTPases. The heterotrimeric G-proteins are composed of three subunits, the catalytic core and the GTP binding sites are located in the α subunit, while the β and γ subunits bind tightly to each other and form a stable protein complex. They are normally associated with, or anchored to, membranes because they act as specific reaction partners of G-protein coupled receptors. In the absence of ligands that bind to the receptor, the GTPase is GDP-bound and thus is

inactive. When the receptor is stimulated by its specific ligand, it acts as a GEF and helps the GTPase to exchange the GDP for GTP. The activated α subunit dissociates from the β and γ subunits and can in turn activate its downstream effectors (*e.g.* ion channels, adenylate cyclase) (Ladds *et al.* 2005; Xue *et al.* 2008). The monomeric small GTPases are proteins homologous to the α subunit of the heterotrimeric G-proteins. They can be bound to membranes but they can also localize in the cytosol. Even though they often need a GEF to be activated, they do not necessary need to be coupled to a transmembrane receptor (Ladds et al., 2005; Takai et al., 2001; Xue et al., 2008).

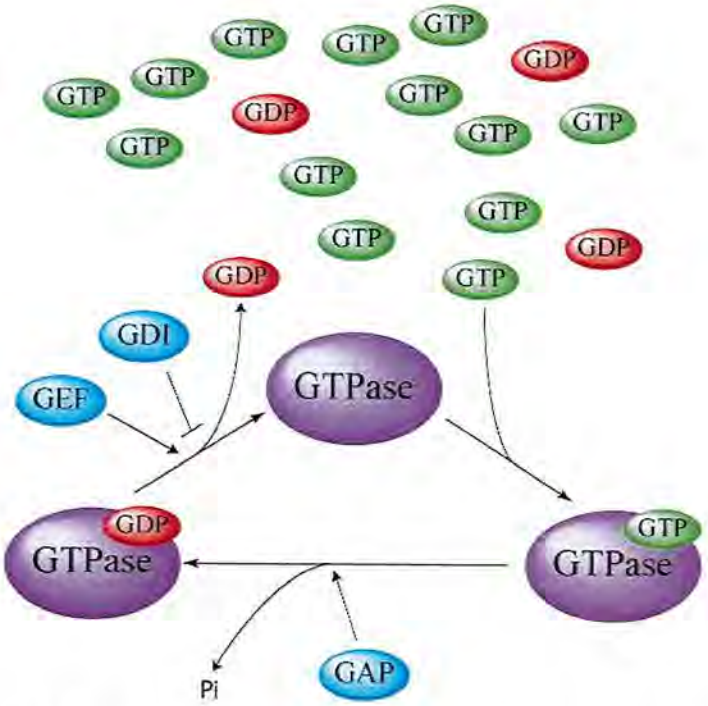

Fig. 1: The GTPase Cycle.
GTPases form a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP) with the help of a GAP. GEF proteins favour release of GDP from GTPases, allowing binding of a new molecule of GTP and re-entry into the cycle, while GDI proteins keep their GTPases bound to a GDP molecule.

Crystallographic studies have revealed three regions of major conformational differences between the GDP- and GTP-bound states of GTPases termed the *switch I-III regions* (Noel et al., 1993; Sondek et al., 1994). These changes are directly linked to the guanine nucleotide binding domain formed by five conserved loops called G1-G5. The G1-G3 boxes are essential for coordination of a Mg^{2+} ion that is crucial for GTP binding, while the G4 and G5 loops are involved in binding to the guanosine ring. Specific point mutations can lead to inactivation or hyperactivation of GTPases. Substitution of the serine (or threonine) within the highly conserved G1 box (within the consensus sequence GXXXXGKS/T) into leucine will render the GTPase unable to coordinate the Mg^{2+} ion

and, consequently, the guanine nucleotides (Farnsworth and Feig, 1991). This nucleotide-free mutant protein is not able to reach the active conformation and, at the same time, binds tightly to and sequesters its GEF. On the other hand, mutation of the conserved glutamine residue in the G3 box (DXXGQ/H/T), which is crucial for GTP hydrolysis, turns the GTPase into a constitutively active GTP-bound protein. Indeed, this allele has an increased affinity for GTP, is GAP-insensitive and displays lower intrinsic GTPase activity, preventing the return to the inactive GDP-bound state. It is important to point out that activation of this allele by a GEF is still necessary (Boguski and McCormick, 1993).

In the next sections, the transduction pathways involved in the sensing of the essential nutrients (glucose, nitrogen and phosphate) will be described to better understand how yeast cells respond at a molecular level to conditions that allow growth.

5.2. Glucose-induced signalling

Yeast cells obtain energy from fermentation (conversion of sugars like glucose, galactose, fructose and others into ethanol) or via respiration (*e.g.* oxidation of a variety of fermentation products like ethanol, lactose or glycerol). Moreover, yeast prefers fermentation over oxidation and glucose or fructose over other fermentable sugars. In other words, if yeast is growing in the presence of all three glucose, galactose and glycerol, it will first ferment glucose, and subsequently galactose into ethanol, and, only lastly, it will oxidize ethanol and glycerol. This hierarchical arrangement is established by allosteric modulation of metabolic key enzymes and by extensive transcriptional regulation networks. Furthermore, addition of glucose to cells growing on non-fermentable carbon sources triggers a rapid change in the pattern of protein phosphorylation and in a massive rearrangement of the transcriptional profile of the genome. More than 40% of the genes alter their expression by more than two-fold within a very short period of time (Gelade et al., 2003). Notably, those changes involve increase in the expression of ribosomal protein genes (to account for the increase in mass generation) and repression of genes required for respiration and metabolism of sugars other than glucose and fructose. The metabolic changes, as consequence of these transcriptional rearrangements, are mediated by several complex molecular networks that regulate interconnected and overlapping processes.

5.2.1. The main glucose repression pathway

The glucose repression pathway is a glucose-induced response that leads to repression of genes encoding proteins involved in the uptake and utilization of alternative carbon sources. The genes repressed by this pathway can be grouped into three main sets. The first group consists of the fructose-1,6-bisphosphatase (*FBP1*) and in the phosphoenolpyruvate carboxykinase (*PCK1*), two enzymes with a unique function for gluconeogenesis. Their repression is sensitive to glucose concentrations as low as 0.01% (Mercado et al., 1994). As *S. cerevisiae* prefers fermentation to respiration, a second group of genes that are silenced encodes mitochondrial enzymes that are needed for the Krebs cycle and for respiration. During fermentation, mitochondria are largely dispensable and thus, genes coding for instance for cytochromes are repressed (Grivell, 1995). The third group of repressed genes codes for proteins needed for the uptake and metabolization of alternative carbon sources or non-fermentable carbon sources, like the MAL proteins (Klein et al., 1996) and the high affinity glucose transporter Hxt2 (Ozcan et al., 1998; Ozcan and Johnston, 1995).

Three main components characterize the glucose repression pathway: the Glc7 protein phosphatase PP1, the serine/threonine kinase Snf1 that is in complex with Snf4, Sip1, Sip2 and Gal83, and the Mig1 transcription complex formed by Mig1, Tup1 and Ssn6, responsible for repression of genes involved in metabolism of alternative carbon sources (Carlson, 1999; Gancedo, 1998). The protein phosphatase 1 needs to associate with regulatory subunits in order to carry out various functions in different processes (not only in the main catabolite repression pathway). In the catabolite repression pathway Glc7 interacts with its regulatory subunit Reg1 (Tung et al., 1992). This complex is believed to be activated by intracellular sugar phosphorylation triggered by Hxk2 (Randez-Gil et al., 1998) and it modulates the kinase activity of Snf1 by regulating its interaction with the Snf1-activating subunit Snf4 (Jiang and Carlson, 1996). Inactive Snf1 cannot phosphorylate Mig1, which thus remains in the nucleus under high glucose levels, exerting repression of transcription of its target genes. At low glucose concentrations, when Snf1 becomes active, Mig1 is phosphorylated and translocates to the cytosol, thereby releasing gene repression (Christensen et al., 2009). On the other hand, it has been reported that Snf1 activity is needed to modulate its own interaction with the Reg1-Glc7 complex (Sanz et al., 2000). After glucose depletion, Snf1 is switched to its active form and it phosphorylates the N-terminal domain of Reg1 thereby recruiting the catalytic subunit (Glc7) to the complex. Upon glucose addition, the PP1 phosphatase dephosphorylates Reg1 and Snf1, converting them into the inhibited form (Sanz et al., 2000) (Fig. 2).

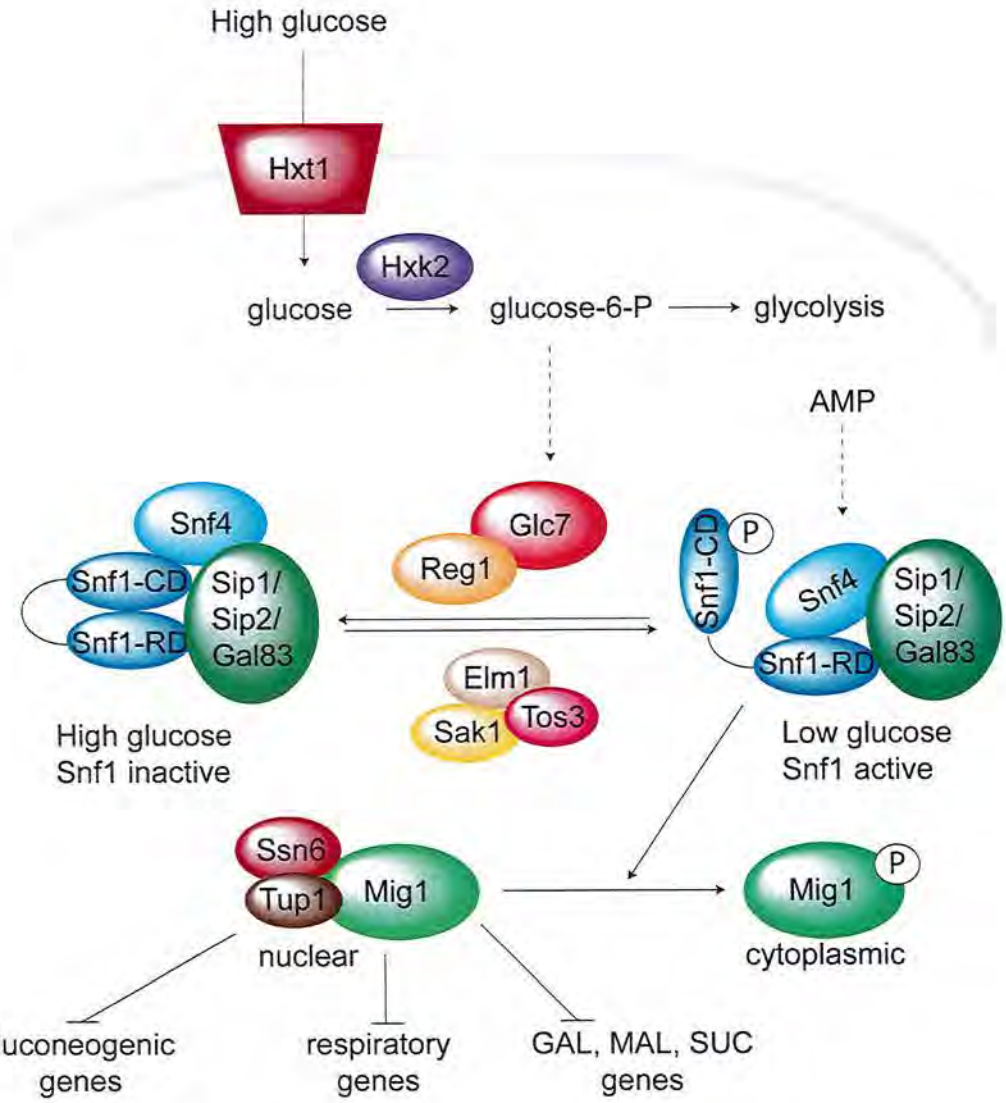

Fig. 2: The Main Glucose Repression Pathway.
The high affinity glucose transporters (Hxt1) transports glucose into the cytoplasm. In this compartment, glucose quickly undergoes glycolysis and is phosphorylated by the the hexokinase 2. This process generates a signal for activation of the phosphatase Glc7 that promotes inactivation of the Snf1 kinase. Under these conditions, Mig1 is dephosphorylated and accumulates in the nucleus to repress genes involved in utilization of alternative carbon sources. Upon glucose depletion Snf1 kinase is activated and phosphorylates the repressor Mig1, which then exits from the nucleus and releases repression of its target genes.

As already mentioned, Snf4 is required for maximal activity of Snf1 in glucose depleted conditions, but other proteins interact with Snf1 and define its target specificity. Gal83 mediates the interaction of the Snf1 complex and Sip4, a transcriptional activator of gluconeogenic genes (Vincent and Carlson, 1999), Sip1, a suppressor of defects caused by reduced Snf1 kinase activity (Yang et al., 1992) and Sip2 that affects cell survival during stationary phase. This happens when cells are starved for glucose (Ashrafi et al., 2000).

Activation of Snf1 is caused by its phosphorylation on threonine 210 triggered by the kinases Pak1, Tos3 and Elm1. It is not clear whether the three kinases are part of a redundant mechanism, or if they are activated by different signals during glucose deprivation. The full activation of Snf1 requires an additional conformational change mediated by Snf4 (McCartney and Schmidt, 2001). The players in the activation of the Snf1 pathway are not clear. Homology studies suggest that an increase in intracellular AMP could somehow lead to the activation of the Snf1 complex (Lutfiyya et al., 1998), but another model postulates that hexokinase 2 may trigger the glucose-sensing function (Hohmann et al., 1999).

The transcription factor Mig1 is responsible for the repression of many genes that need to be silenced during growth on glucose or fructose, for example those acting during the initial steps in the utilization of alternative carbon sources (Klein et al., 1996). Under conditions of high extracellular glucose Mig1 acts also in the downregulation of some intermediate affinity glucose transporters like Hxt2 and Hxt4 by binding to their promoter with the co-repressor proteins. Mig1 activity is dependent on its localization: in the active form, it is present in the nucleus, but when fermentable sugars are depleted, a Snf1-dependent phosphorylation leads to the dissociation of Mig1 from its co-repressor and triggers its translocation to the cytoplasm (Vallier and Carlson, 1994). Mig1 interacts also with Hxk2 that might inhibit Snf1 kinase activity preventing Mig1 phosphorylation and subsequent translocation inside the nucleus (Moreno et al., 2005). Since the protein kinase Snf1 is active in glucose depleted conditions and is required for the expression of all the genes that are downregulated in the presence of glucose, *snf1*Δ mutants are unable to grow on non-fermentable carbon sources.

5.2.2. The G-protein Ras2 and the Ras/cAMP pathway

The *Saccharomyces cerevisiae* Ras1 and Ras2 proteins are two small (approximately 34 kDa) monomeric G-proteins that are homologues of the mammalian proto-oncogene H-Ras (DeFeo-Jones et al., 1983). Both Ras proteins are involved in glucose sensing and signal transduction in yeast.

The GTP/GDP ratio on the Ras proteins is mostly controlled by the activators Cdc25 and Sdc25 that have a GEF activity, and by the inhibitor proteins, Ira1 and Ira2, that have a GAP activity. The Ira proteins improve the low intrinsic ability of Ras to hydrolyse the GTP in GDP (Fig.3). Their activity is controlled by the intracellular pH, and both proteins become inactive when the cytoplasm acidifies (Colombo et al., 1998). The $Ras2^{Val19}$ mutant, which is impaired in its GTPase activity and is constitutively in the activated form bound to GTP, is also insensitive to regulation by the Ira

proteins (Kataoka et al., 1984). Cells carrying this mutation show the typical phenotype associated with an overactive Ras protein like high heat shock sensitivity and reduced cell viability (Tanaka et al., 1989).

Addition of glucose to derepressed or stationary cells triggers a very fast and transient increase in the GTP/GDP ratio on Ras2. Activated Ras2, which is membrane bound by its farnesyl group, in turn, stimulates the plasma membrane-localized adenylate cyclase (Cyr1/Cdc35). This process results in a transient production of cAMP from ATP leading to a boost in the activity of the cAMP-dependent protein kinase PKA. The mechanism of activation of the Ras/cAMP pathway by glucose is not completely clear but it is known that glucose or fructose needs to be transported inside the cell and phosphorylated by the sugar kinases Hxk1, Hxk2 or by the glucose specific kinase Glk1 (Rolland et al., 2000). Knowledge concerning the link between glucose addition and activation of adenylate cyclase via Ras1/2 is missing. Possibly, intracellular sensing of a fermentable sugar somehow activates the GEF Cdc25. Indeed, it has been shown that the increase in the ratio of GTP/GDP bound to Ras2 depends on glucose phosphorylation and on Cdc25 (Colombo et al., 2004). However, the same authors also confirmed that intracellular acidification and inhibition of the Ira proteins affect the Ras2 GTP/GDP ratio and thus, it cannot be excluded that glucose metabolism, which affects the acidity of the cytosol, may be important as well.

In order to yield a complete activation of the Ras/cAMP pathway, glucose has to be detected also extracellularly through a glucose sensitive *G-p*rotein *c*oupled *r*eceptor (GPCR) system composed of the G-protein, Gpa2, and its coupled receptor, Gpr1 (Colombo et al., 1998; Dechant and Peter, 2008). When glucose is present in the extracellular environment it binds to Gpr1 which, in turn, results in loading GTP on Gpa2 allowing it to stimulate adenylate cyclase to produce cAMP (Kraakman et al., 1999) (Fig. 3). It is important to note that glucose is unable to induce a cAMP signal in cells expressing the $Ras2^{Val19}$ mutant, probably because this allele constantly stimulates Cyr1/Cdc35 and saturates the signal through this pathway. This has two distinct consequences: on one hand, constitutive stimulation of PKA by cAMP triggers a high feedback inhibition loop (see below) with high phosphodiesterase activity, on the other hand, Gpa2 fails in this context to further stimulate adenylate cyclase in response to glucose (Colombo et al., 1998).

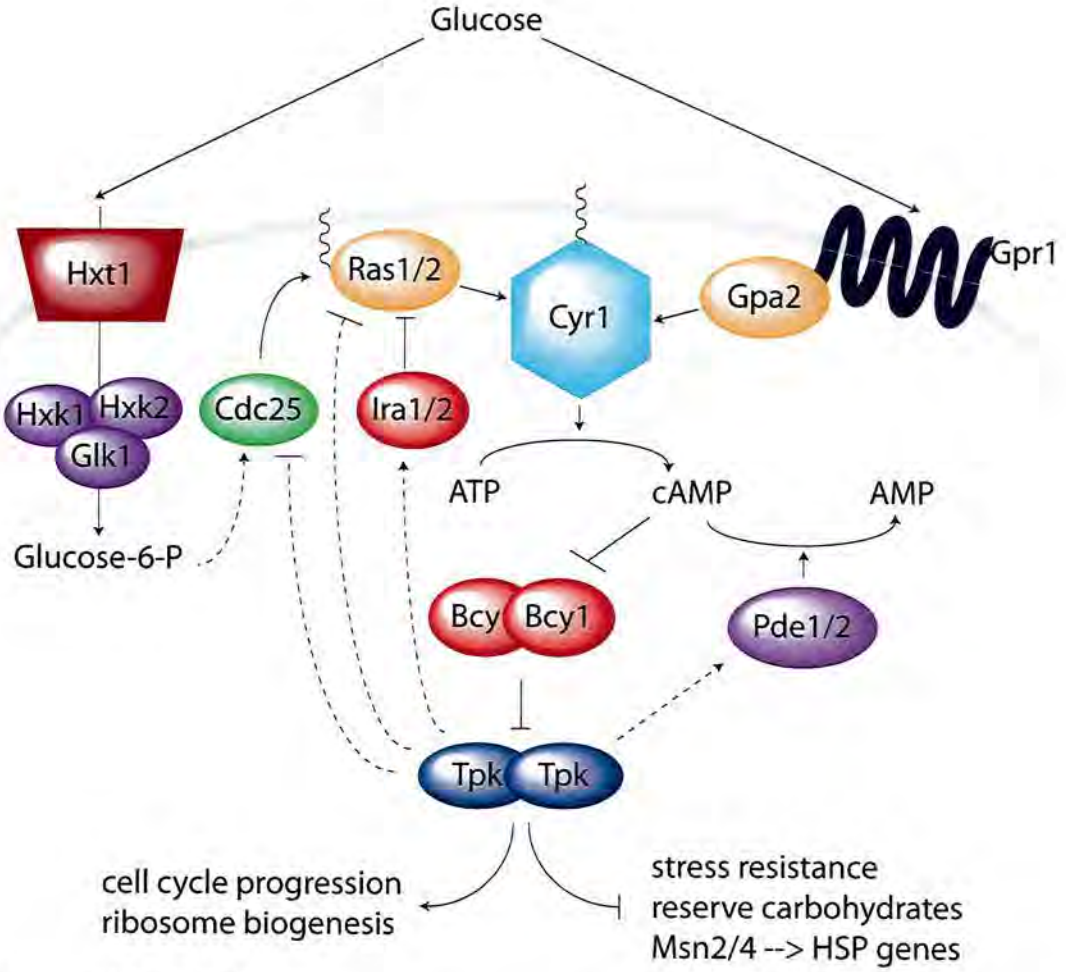

Fig. 3: The Ras/cAMP Pathway.
cAMP is a well studied second messenger important for PKA activation upon glucose sensing. Glucose can be sensed by the GPCR system in the extracellular environment or following its intracellular metabolisation. Phosphorylation of glucose by glucose kinases generates a signal that activates Ras GTPases Ras1 and Ras2, which in turn stimulate Cyr1/Cdc25, in parallel to Gpa2, for cAMP production. cAMP favours activation of PKA, which stimulates growth, represses the stress response and regulates the whole pathway via different feedback mechanisms. Dotted lines are putative interactions.

As mentioned above, cAMP activates the cAMP-dependent protein kinase PKA, which is composed of three catalytic subunits encoded by the genes *TPK1*, *TPK2* and *TPK3* and the regulatory subunits encoded by *BCY1* gene. Two monomeric catalytic subunits of PKA interact with a Bcy1 dimer during an inhibited state. cAMP binds to the two Bcy1 subunits thereby releasing the catalytic subunits, which can, for instance, phosphorylate the transcription factors Msn2 and Msn4 affecting their localization (Gorner et al., 1998). PKA activation results in a wide range of changes at both transcriptional and post-translational levels. For instance, high activity of PKA triggers nuclear export of Msn2/4 and thus repression of stress-resistance genes (*HSP* genes). Additionally, activation of PKA causes repression of the invertase 2 (*SUC2*) gene, induction of ribosomal protein genes (*RPL25* and *RPS33*), post-transcriptional activation of trehalase (Thevelein, 1984) and

phosphofructokinase 2 (Francois et al., 1984) and inhibition of fructose 1,6-bisphosphatase (Mazon et al., 1982).

A very potent mechanism down-regulating cAMP levels is feedback inhibition of cAMP synthesis by PKA (Nikawa et al., 1987). This involves the activation of the low affinity phosphodiesterase Pde1 that degrades cAMP into AMP (Fig. 3). Another mechanism proposed is the phosphorylation of the Ras proteins by PKA that causes a decrease in their ability to interact with adenylate cyclase (Resnick and Racker, 1988). The possibility of Ras2 to be a target for feedback inhibition was also proposed more recently by Colombo *et al.* (2004). Cdc25 was also shown to be phosphorylated in response to glucose, resulting in an impaired ability to interact with Ras (Gross et al., 1992). The Ira proteins have been proposed as possible targets for feedback inhibition after glucose induced stimulation, as they contain consensus sequences for phosphorylation by PKA (Tanaka et al., 1989). Finally, also the membrane/cytoplasmic localization of adenylate cyclase is affected by the activity of PKA (Engelberg et al., 1990; Mitts et al., 1990). Importantly, none of the proteins mentioned above have been surely demonstrated to be the *in vivo* target of the feedback inhibition mechanism controlled by PKA.

5.2.3. The Ras2/MAP kinase pathway

Ras2 is also the activator of another important pathway: the MAPK pathway that leads to pseudohyphal differentiation. Pseudohyphae consist of filamentous cells that are still connected to each other and that form rough-edged colonies. This kind of growth is a peculiarity of diploid cells. Haploids in the same conditions undergo a different kind of growth called invasive growth where cells invade the solid medium. Both pseudohyphal and invasive growth modes are induced by carbon and nitrogen deprivation and depend on the genetic background. These growth forms may be valid alternatives to growth arrest and spore formation as they are believed to allow cells to search for novel nutrient sources.

Activated Ras2 stimulates the guanine nucleotide exchange factor Cdc24 which, in turn, facilitates loading of GTP on Cdc42, another monomeric GTPase involved also in maintainance of cell polarity (Adams et al., 1990). The GTP-bound form of Cdc42 displaces the negative regulator Hsl7 from the kinase Ste20 thereby triggering the first reaction of a MAP kinase cascade. The pathway further comprises three kinases: the MEKK or MAPKKK Ste11, the MEK or MAPKK Ste7, and the MAPK Kss1. When Kss1 is phosphorylated by Ste7, both on a threonine and on a tyrosine residue, it is able to phosphorylate Ste12 and Dig1/2; the Dig proteins dissociate from Ste12 and

this allows derepression of the target genes to which Ste12 binds (Bardwell et al., 1998) (Fig. 4). Those genes have a common region in their promoters that is called FRE (*f*ilamentation and invasive *r*esponse *e*lement). These are composite elements with two adjacent binding sites for the transcription factors Ste12 and Tec1 which bind cooperatively to the consensus sequences TGAAACA and CATTCT/C in the FRE element, respectively.

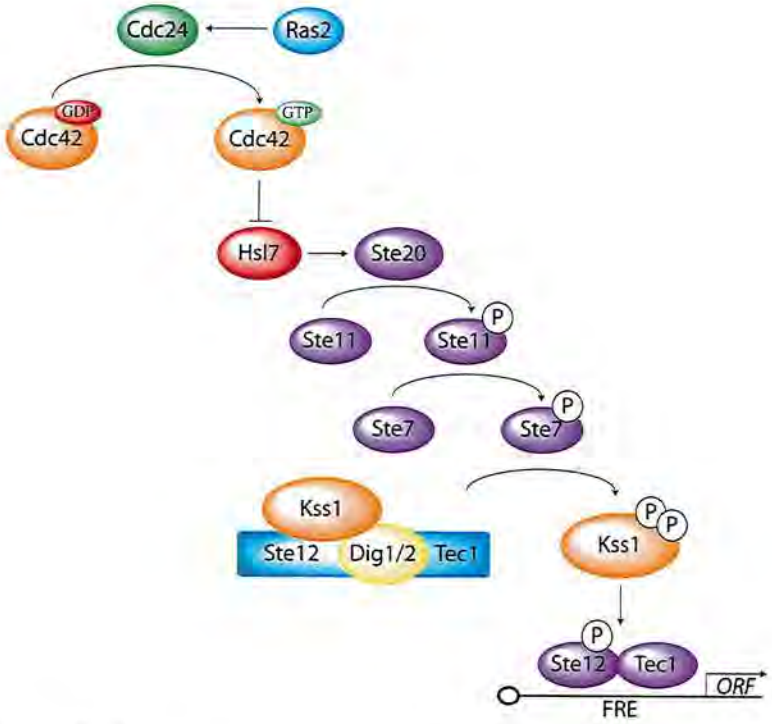

Fig.4: The MAP Kinase Pathway.
External glucose activates Ras2, which, in turn, through a series of intermediate steps, causes the sequential phosphorylation of the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Kss1. Ste12 is activated by Kss1-dependent phosphorylation and activates transcription of genes with the FRE element in their promoter.

The role of the Ras2/cAMP pathway in pseudohyphal and invasive growth is not clear and there are discordances regarding the role of PKA: Robertson and Fink did not observe any effect when *TPK1* was deleted, but Pan and Heitman reported that Tpk1 had a repressing role on pseudohyphal and invasive growth. Nevertheless, it appears to be clear that deletion of *TPK2* blocks pseudohyphal and invasive growth, while deletion of *TPK3* stimulates both processes (Robertson and Fink, 1998). Furthermore, Tpk2 interacts *in vitro* with Sfl2 and Mga1 that contain both a helix-turn-helix DNA binding-motif, which was first described in the heat-shock transcription factor Hsf1 (Fujita et al., 1989), and phosphorylation consensus sites for Tpk2. Deletion of *SFL2* enhances pseudohyphal and invasive growth, while deletion of *MGA1* does not interfere with formation of elongated cells during nitrogen starvation, but it causes a large increase in the proportion of cells budding randomly (Lorenz and Heitman, 1998). The downstream targets of these proteins in the regulation of

pseudohyphal growth have not yet been identified, but the *MUC1/FLO11* gene could be a good candidate. Consistently, *MUC1/FLO11* mRNA levels are increased in a *sfl1* mutant and a *sfl1Δ muc1Δ* homozygous diploid is completely described in filamentation. Further experiments carried out in a *bcy1Δ* strain showed that the increase in the expression of *MUC1/FLO11* genes necessarily depends on the protein Flo8 (Kobayashi et al., 1996), a known target of PKA (Pan and Heitman, 1999). The fact that a double mutant *sfl1Δ tpk2Δ* shows no defect in filamentation (Robertson and Fink, 1998), suggests that the activation of Flo8 is not strictly dependent on Tpk2 and can also be performed by other upstream effectors.

The importance of the cAMP-dependent pathway for morphogenesis is highlighted by the fact that external cAMP stimulates pseudohyphal and invasive growth (Kubler et al., 1997), while both growth modes are blocked by overexpression of the phosphodiesterase Pde2 (Ward et al., 1995). The Ras2/cAMP pathway can play a role in stimulating invasive growth through the suppression of the activity of the stress-response transcription factors Msn2 and Msn4. Indeed, invasive growth cannot take place in a *ras2Δ* strain, but it is restored in a *ras2Δ msn2Δ msn4Δ* triple mutant (Stanhill et al., 1999). Therefore, one or more genes activated by Msn2/4 may be negative regulators of invasive growth.

5.3. Phosphate metabolism and transport

Inorganic phosphate (Pi) is an essential nutrient for all organisms, being required as structural component of nucleic acids and phospholipids, and as a critical element for other cellular metabolites such as, for instance, ATP and glucose-6-phosphate. Maintaining proper phosphate homeostasis is therefore critical to ensure survival of cells and organisms. Besides its nutrient function, Pi exerts an important signalling function, regulating not only its own transport and metabolism, but also a various properties related especially to the growth rate of the cell. Notably, phosphate starvation triggers cell cycle arrest and entry into a G_0-like state (Lillie and Pringle, 1980).

Saccharomyces cerevisiae, as many other living organisms, uses preferentially Pi as phosphate source. When extracellular Pi becomes limiting, extracellularly secreted acid phosphatases can hydrolyse phosphate units from a broad spectrum of phosphor-ester substrates, thereby increasing the available Pi in the medium (Bostian et al., 1980). In addition, yeast can utilise alternative phosphate sources, like glycerophosphoinositol and glycerophosphocholine (Almaguer et al., 2004).

Once transported inside the cell, Pi is incorporated into numerous compounds and metabolites. A major fate of free cellular Pi consists of the incorporation into inorganic polyphosphate (polyP), a linear polymer that is stored predominantly in the vacuole consisting of a few to several hundreds of Pi residues linked together by high-energy phosphoanhydride bonds (Kulaev and Kulakovskaya, 2000). The vacuolar pool of polyP plays an important role in the regulation of phosphate homeostasis and survival of yeast cells under conditions of limiting Pi (Shirahama et al., 1996).
Low inorganic phosphate concentration induces the expression of nonspecific scavenger phosphatases, such as Pho5 (Carroll and O'Shea, 2002). Expression of *PHO5* is regulated by the basic helix-loop-helix transcription factor Pho4 that is phosphorylated by Pho85, a yeast cyclin-dependent protein kinase (CDK). Utilizing Pho80 as its cyclin partner, Pho85 phosphorylates Pho4 at multiple sites that keep it inactive and in the cytoplasm, thereby blocking transcription of *PHO5* (Kaffman et al., 1994). The activity of Pho80-Pho85 is controlled by the CDK inhibitor Pho81 that is repressed and inactivated by high phosphate. However, no evidence for binding of phosphate to any of the components of the system has been obtained and the primary sensing event remains unknown. It has been proposed that the phosphate sensing machinery is activated by plasma membrane proteins (like Pho84 and Pho87) that mediates both sensing and transport across the membrane of inorganic phosphate (Giots et al., 2003; Holsbeeks et al., 2004).

5.4. Amino acid sensing and transport

Yeast cells need to use amino acids for translation and, in case it is required by growth conditions, also for alternative nitrogen sources. Amino acids can be *de novo* synthesized or, in case of growth in rich conditions, can be taken up from the medium by specific or general permeases. Different permeases are regulated and sorted to the plasma membrane in response to certain environmental stimuli. The high-capacity general amino acid permease Gap1, for instance, is regulated by the nitrogen source present in the medium (Magasanik and Kaiser, 2002). *GAP1* is target of the *n*itrogen *c*atabolite *r*epression (NCR) pathway (see below) (Cooper and Sumrada, 1983) and Gap1 post-translational modifications (*e.g.* sorting to the plasma membrane) are sensitive to rapamycin (De Craene et al., 2001). There are other permeases that are regulated by the plasma membrane SPS (Ssy1-Ptr3-Ssy5) sensor system that functions as a sensor of extracellular amino acids (Forsberg and Ljungdahl, 2001; Klasson et al., 1999). Amino acid sensing is initiated by amino acid binding to Ssy1, which transmits signals intracellularly through Ptr3 and Ssy5. Ssy5 is a protease that activates by cleavage two transcription factors named Stp1 and Stp2 allowing them to enter the nucleus and activate the expression of target genes encoding amino acid permeases such as Gnp1

and Apg1 (Liu et al., 2008). Ptr3 needs to be highly phosphorylated probably by Yck1 and Yck2 (recruited by Ssy1) for full activation (Spielewoy et al., 2004), while inactivation in the absence of external amino acids is triggered by the Rts1/PP2A phosphatase complex and likely controlled by TORC1 (Eckert-Boulet et al., 2006). Moreover, TORC1 has been also recently reported to control the degradation of the transcription factor Stp1. Addition of rapamycin triggers rapid disappearance from the nucleus and Sit4-dependent degradation of Stp1-GFP. On the other hand, expression levels of Stp1 affect the cells' sensitivity towards rapamycin probably because higher levels of Stp1 cause higher uptake of amino acids, which are important for TORC1 activity (Shin et al., 2009). Interestingly, external leucine is reported to be the most important amino acid for activation of the SPS sensor (Gaber et al., 2003; Liu et al., 2008) (Fig. 5).

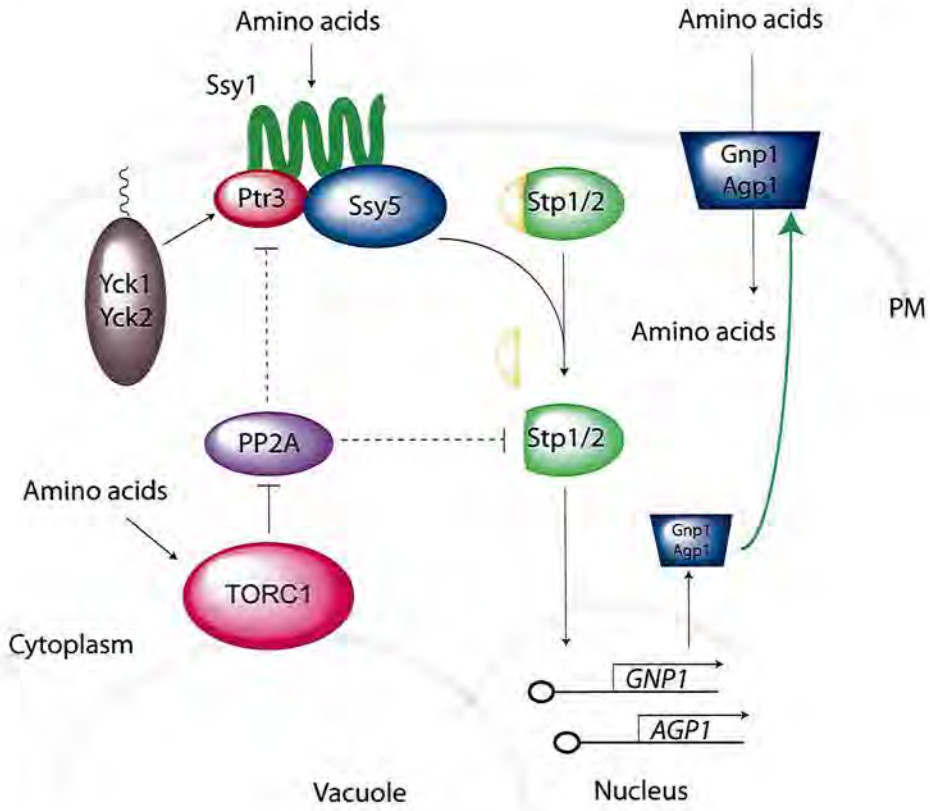

Fig. 5: The SPS Sensor System.
When amino acids are present in the extracellular environment they are sensed by the plasma membrane receptor Ssy1. The signal is then transmitted across the plasma membrane to Ptr3 and then Ssy5 that process the transcription factors Stp1 and Stp2 that, in turn, enter the nucleus and start transcription of the genes coding the amino acid permeases Gnp1 and Agp1 that are subsequently sorted to the plasma membrane.

5.5. The retrograde (RTG) response pathway

Yeast cells can assimilate nitrogen condensing a molecule of ammonium with α-ketoglutarate in order to form glutamate. Thus, they need α-ketoglutarate to properly assimilate nitrogen during growth in ammonia containing media (Butow and Avadhani, 2004). In turn, in cells with mitochondrial dysfunctions or that are growing on glucose, the citric acid cycle is repressed and α-ketoglutarate must be formed from pyruvate and acetyl-CoA by an anapleurotic pathway (Zaman et al., 2008). Under these conditions, the three enzymes Cit1 (cytrate synthase), Aco1 (aconitase) and Idh1 (isocitrate dehydrogenase) must be consequently active in order to satisfy the demand of glutamate, a precursor of other amino acids. Those genes are upregulated, together with other two sets of genes, thanks to the retrograde (RTG) pathway (Liu and Butow, 1999). The RTG regulatory pathway consists of four positive regulators, Rtg1, Rtg2, Rtg3 and Grr1 (Liu and Butow, 1999; Liu et al., 2005) and four negative regulators, Mks1, Bmh1, Bmh2 and Lst8 (Dilova et al., 2002; Giannattasio et al., 2005). Rtg1 and Rtg3 interact with each other forming a transcriptional activator of the RTG target genes (Jia et al., 1997). The nuclear localization of the heterodimer is controlled by the other members of the pathway. When mitochondria are functional, Rtg1 and Rtg3 are kept in the cytoplasm by the Mks1-Bmh1-Bmh2 complex, but upon mitochondrial disruption, Rtg2 sequesters Mks1 and Rtg1-Rtg3 heterodimers are quickly relocalized in the nucleus where they can start transcription of the target genes (Sekito et al., 2000). The RTG pathway is also under the negative control of TORC1 since Rtg1/3-dependent gene expression is activated in rapamycin treated cells (Komeili et al., 2000). Lst8, a subunit of TORC1, acts via an unknown mechanism as inhibitor of the RTG pathway and its action is thereby believed to be controlled by TORC1.

5.6. Nitrogen and TOR pathway

Nitrogen is an essential nutrient for yeast. Nitrogen starvation is able to trigger different developmental responses according to the cell type and environmental cues. In diploid cells, nitrogen starvation induces meiosis and sporulation (Herskowitz, 1988), while, in haploid cells of particular yeast backgrounds (Liu et al., 1996), it triggers a differentiation program called invasive growth (Werner-Washburne et al., 1996). In other backrounds, like the BY4741, nitrogen starvation triggers cell cycle arrest and entry into quiescence.

Nitrogen is a major component of complex macromolecules (*e.g.* proteins) and yeast can obtain it from a wide variety of sources like glutamine, glutamate, ammonium, urea and amino acids like

proline, ornithine and allantoin (ter Schure et al., 2000). Ammonium, glutamate and glutamine are considered to be preferred nitrogen sources. Ammonium is assimilated via the formation of glutamate and glutamine which are important nitrogen precursors for the formation of essential organic compounds like amino acids and nucleotides (Courchesne and Magasanik, 1988). Glutamate can be synthesised by the the glutamate dehydrogenase Gdh1 which adds a molecule of ammonium to a molecule of α-ketoglutarate. In turn, glutamate can be converted into glutamine by the essential enzyme glutamine synthase Gln1, which incorporates another molecule of ammonium. The activities of Gln1 and Gdh1 depend on the nitrogen source present in the medium. When a good nitrogen source (ammonium or glutamine for instance) is present, yeast cells preferentially express Gdh1 and Gln1 and represses the transcription of genes encoding proteins involved in the uptake and metabolism of poor nitrogen sources (urea and proline for instance). This response is called *n*itrogen *c*atabolite *r*epression (NCR) (Cooper and Sumrada, 1983). When cells are shifted from good to poor nitrogen sources or under conditions of nitrogen starvation, the NCR pathway is activated and NCR-responsive genes are induced (Daugherty et al., 1993). The protein that act as nitrogen sensor is still unknown, but it has been shown that the *t*arget *o*f *r*apamycin (TOR) pathway plays a role in controlling the NCR pathway and in coordinating growth with environmental cues.

TOR (*t*arget *o*f *r*apamycin) was originally identified genetically by mutations in yeast, *TOR1–1* and *TOR2–1*, that confer resistance to the growth-inhibitory properties of the FKBP (FK506 binding protein)-rapamycin complex (Heitman et al., 1991). The *TOR1* and *TOR2* genes encode the two large and highly homologous conserved, Ser/Thr kinases Tor1 and Tor2. Both Tor proteins contain a C-terminal region with strong homology to the catalytic domain of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinase (Kunz et al., 1993), shared by a large family of proteins in several organisms. Tor proteins also contain up to 20 tandemly repeated HEAT (*H*untington, *E*longation Factor 3, PR65/*A*, *T*OR) motifs at their N-terminus (Andrade and Bork, 1995), roughly grouped into two blocks that, together with the nearby localized FAT domain, were thought to be necessary for protein-protein interactions, indicating thus that Tor1 and Tor2 could also serve as scaffolds within large protein complexes.

Rapamycin treatment rapidly inhibits growth and triggers complete cell cycle arrest within one cell cycle (Heitman et al., 1991). Early findings indicated that mammalian TOR (mTOR) activates translation initiation in response to nutrients via inhibition of the eIF4E inhibitor 4E-BP1 (Hara et al., 1998). The same mechanism had also been proposed to occur in yeast (Barbet et al., 1996). Later on, yeast and mammalian TOR have been demonstrated to control several additional readouts,

all of which are related to cell growth. These readouts include control of stability and localization of permeases (Schmidt et al., 1998), autophagy (Noda and Ohsumi, 1998), PKC signaling (Ziegler et al., 1999), ribosome biogenesis (Powers and Walter, 1999), transcription (Mahajan, 1994), and, although more a consequence than a readout, cancer (West et al., 1998).

Tor1 is not essential, but a *tor1Δ tor2Δ* double mutant uniquely mimics the cell cycle (G_1/G_0) arrest typically observed following of rapamycin exposure of cells. On the other hand, disruption of Tor2 alone is lethal but does not cause cell cycle arrest in a specific phase. This is because Tor2, in addition to its redundant function with Tor1 in a rapamycin-sensitive signalling pathway, also has a rapamycin-insensitive function that Tor1 cannot perform (Helliwell et al., 1994). Additionally, the fact that *TOR1-1* cells are able to grow in the presence of rapamycin suggested that the Tor2-specific functions are rapamycin-insensitive (Zheng et al., 1995). TOR depletion studies have shown that this Tor2 unique function mediates the cell cycle-dependent polarization of the actin cytoskeleton controlling the *c*ell *w*all *i*ntegrity (CWI) pathway (Schmidt et al., 1996). A polarized actin cytoskeleton orients the secretory pathway toward a discrete growth site (the bud) and thereby determines the spatial growth pattern of yeast cells. Rapamycin-insensitive TOR signaling has also been detected in mammalian cells (Sarbassov et al., 2004). mTORC2 shows functional similarities with its yeast counterpart, for instance, mTORC2 appears to regulate actin structures via RHO family GTPases and PKCα.

In yeast, several comprehensive studies have been carried out by different groups to identify binding partners of Tor1 and Tor2. The procedures relied on functional, epitope-tagged versions of Tor1 and Tor2 expressed at endogenous levels, and involved immunoprecipitation (Fadri et al., 2005; Loewith et al., 2002; Reinke et al., 2004). From those studies it appeared that in yeast there are two distinct Tor complexes termed TORC1, composed of either Tor1 or Tor2, Tco89, Lst8 and the yeast ortholog of raptor, Kog1, and TORC2, composed by Tor2 only but including six other proteins (Avo1, Avo2, Avo3, Bit2, Bit61 and Lst8). It has been proposed that the two complexes TORC1 and TORC2 control a variety of processes related to cell growth. These processes can be formally divided into two major control functions: 'temporal' and 'spatial' control of cell growth. 'Temporal' control refers to TORC1-regulated translation, transcription, ribosome biogenesis, nutrient import and autophagy, readouts that collectively determine cell mass accumulation in response to changing nutrient conditions. 'Spatial' control refers to the cell cycle and TORC2-dependent regulation of the actin cytoskeleton, a prerequisite for establishing cell polarity (Martin and Hall, 2005).

Different studies used mass spectrometry to identify proteins that coprecipitated with mTOR from mammalian cell extracts. Crucial to their success was the method of sample preparation prior to immunoprecipitation. Sabatini and coworkers thought that mTOR-containing complexes might be prone to dissociate in buffer containing nonionic detergent. To avoid this dissociation, they added a reversible chemical crosslinker to their lysis buffer to stabilize interactions between mTOR and putative partner proteins. This strategy yielded a novel 150 kDa polypeptide, which they termed *r*egulatory *a*ssociated *p*rotein of m*TOR* (raptor) (Kim et al., 2002). Human raptor contains a unique amino-terminal "raptor N-conserved" (RNC) region followed by three HEAT repeats and seven WD40 repeates that form a β propeller structure important to mediate protein-protein interactions (Li and Roberts, 2001). Thus, the domain structure of raptor is consistent with a role as an adaptor in multiprotein complexes. To show that raptor is a critical regulator of mTOR function, the same group used small-interfering RNA (siRNA) technology to knock down raptor expression in human cells. The phenotypic consequences of raptor deficiency (*e.g.* inhibition of amino acid-induced S6K1 activation and a reduction in cell size) were qualitatively similar to those induced by either siRNA-mediated mTOR depletion or treatment with rapamycin (Kim et al., 2002). Using different biochemical assays (co-immunoprecipitation and kinase assay) it has been found that mTOR phosphorylates 4E-BP1 and S6K1 and binds to them via the raptor subunit (Hara et al., 2002). Additional results supported a model in which raptor functions as a bridging protein that presents 4E-BP1 and S6K1 (and possibly additional substrates) to the mTOR kinase domain for phosphorylation. The substrate-scaffolding model nicely explains the earlier finding that nonionic detergents inhibit 4E-BP1 phosphorylation by mTOR; since these detergents dissociate raptor, they block the raptor-dependent presentation of 4E-BP1 to mTOR. mTORC1 contains also mLst8 which shares 28% of identity with the yeast Lst8. However, the function of mLst8 has not been clarified yet although it seems to be crucial for the kinase activity of mTOR (Harris and Lawrence, 2003). PRAS40 is another mTORC1-interacting protein that was recently identified. It was proposed that PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding competing with 4E-BP1 for binding to raptor (Wang et al., 2007).

Since the TORC2 and the 'spatial' control of cell growth are less relevant for the purposes of this work, they will be shortly described in this section. The TORC1 network will be extensively described in the following section. Cell polarity is an importantaspect of cell organization and it is most evident in specialized cell types, such as epithelial, secretory, or motile cells. In fact, all cells exhibit this capacity, with the most basic manifestation of this process being cell division, or

budding in the case of *S. cerevisiae.* Cell polarity, as reflected by polarized growth and organelle segregation during cell division in yeast, appears to follow a simple hierarchy. On the basis of physical cues from previous cell cycles or stochastic processes, yeast cells select a site for bud emergence that also defines the axis of cell division toward which they recruit signaling molecules. After the initial bud emergence, further cell growth is restricted to the bud as the cell orchestrates the duplication and segregation of its organelles. During mitosis and cytokinesis, secretion is directed to the bud neck to lay down a septum, separating the mother and daughter (Pruyne et al., 2004). Polarized growth is directed by an underlying polarization of the actin cytoskeleton: actin cables orient from the mother cell to the bud and cortical actin patches concentrate in the bud.

The involvement of TORC2 in actin organization was initially suggested by the fact that overexpression of *CCT6* (gene encoding a chaperonin involved in the folding and assembly of actin structures) suppresses the lethality and the defects in polarized organization of the actin cytoskeleton of a *tor2*Δ mutant, but not the lethality of a *tor1*Δ *tor2*Δ double mutant (Schmidt et al., 1996). Tor2 signals to the actin cytoskeleton by activating a Rho-type GTPase switch. More specifically, Tor2 activates the GDP/GTP exchange factor Rom2 (Schmidt et al., 1997). Rom2 in turn converts Rho1 and Rho2 to an active, GTP-bound state. GTP-bound Rho binds and activates Pkc1 which then signals to the actin cytoskeleton via a MAP kinase cascade composed of Bck1, Mkk1, and Mpk1/Slz2 (Helliwell et al., 1998a; Helliwell et al., 1998b; Schmidt et al., 1997). Hyperactivation of components of the CWI (*c*ell *w*all *i*ntegrity) pathway suppresses, like Cct6 overproduction, defects caused by mutations in *TOR2*. A direct link between TORC2 and Rom2 remains to be shown (Fig. 5).

Ypk1 and Ypk2 are two redundant protein kinases involved in cell wall integrity. Like loss of TORC2 function, loss of YPK function can be suppressed by overexpression of components of the CWI pathway (Schmelzle et al., 2002). Moreover, hyperactivation of Ypk1 or Ypk2 was shown to suppress the lethal phenotype of *torc2* mutants, raising the possibility that YPKs and TORC2 may function in the same pathway. *In vivo* experiments showed indeed that Ypk2 activity is modulated via direct phosphorylation by TORC2 (Kamada et al., 2005), leading to the hypothesis that TORC2 might signal to the CWI components via Ypk2.

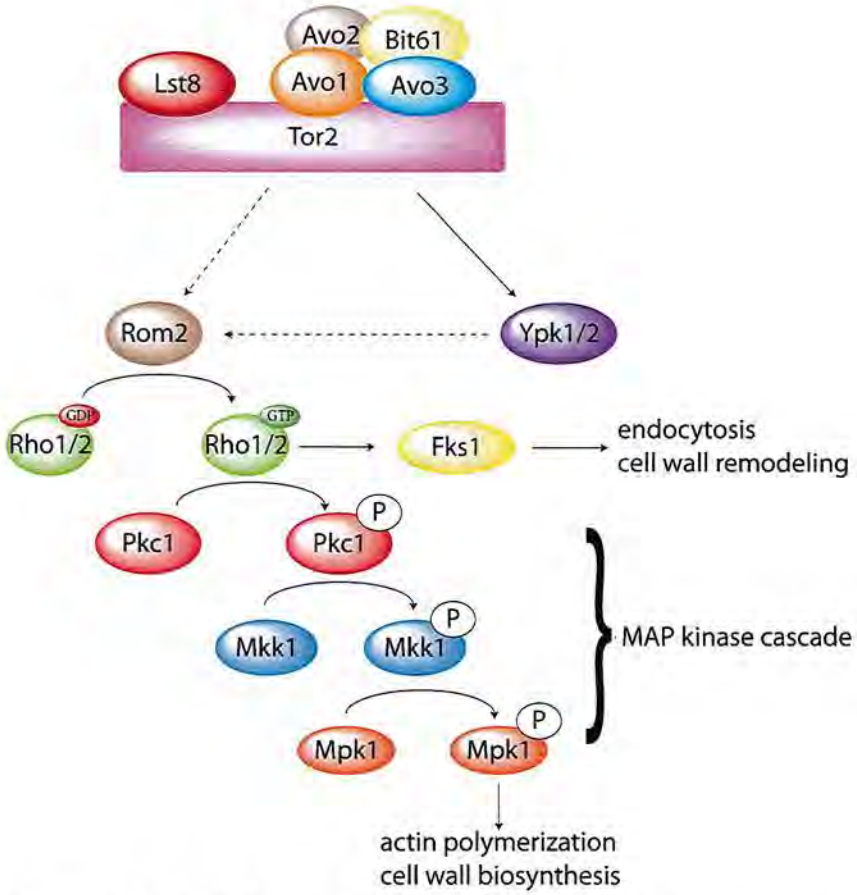

Fig. 6: TORC2 Controls Cell Wall Biosynthesis and Endocytosis.
TORC2 in yeast is composed of Tor2, Avo1-3, Bit61 and Lst8 and is rapamycin-insensitive. The GEF Rom2 is activated by TORC2 (directly or via Ypk1/2) and stimulates the activation of the Rho GTPases. Rho1 and Rho2 stimulate endocytosis via Fks1 and trigger a MAP kinase cascade ending with Mpk1, which promotes both actin polymerization and cell wall biosynthesis. Dotted lines represent putative interactions.

The internalization of plasma membrane proteins into the endocytic pathway is a key regulatory event in signal transduction, nutrient uptake, and ion homeostasis. The activity and interaction of the network of proteins and lipids that comprise the endocytic machinery is carefully controlled in response to intra- and extracellular signals, in particular lipids and lipid kinases. For instance deletion of *lcb1* gene, which codes for a protein involved in the first step of sphingolipid synthesis, results in an endocytosis mutant phenotype. Inactivation of protein phosphatase 2A or overexpression of either the Pkc1 or Yck2 kinase, is able to restore endocytosis in *lcb1* mutants suggesting that Yck2 kinase activity is essential for endocytosis (Friant et al., 2000). In turn, efficient endocytosis is dependent on proper actin cytoskeleton and, consequently, on activity of Rho1 that controls receptor internalization via Fks1 (Fig. 6).

Recent findings significantly extended the understanding of the regulation of sphingolipid pools by TORC2 by demonstrating that a functional Avo3 protein, an essential and specific component of

TORC2, is required for maintenance of normal levels of ceramides in vivo. This, in turn, allows for the synthesis of more complex sphingolipids that are required for cell growth (Aronova et al., 2008; Tabuchi et al., 2006). Since endocytosis has been proposed to be controlled also by sterol and sphingolipid pools (Dickson et al., 2006), it might be reasonable to speculate that TORC2 controls this process also via regulation of sphingolipid biosynthesis.

5.6.1. TORC1 readouts

TORC1 in yeast is composed of either Tor1 or Tor2, Kog1, Tco89 and Lst8. It is rapamycin sensitive and controls the "temporal" aspects of cell growth in response to nutrients. The processes controlled by TORC1 will be described in the next sections.

5.6.1.1. Control of translation

A critical insight into TORC1 function was made with the observation that rapamycin treatment elicits a rapid and pronounced inhibition of translation initiation (Barbet et al., 1996). This mechanism is well characterized in both yeast and mammals. The kinase Sch9 (yeast ortholog of S6K) has been recently shown to be a direct target of TORC1 in yeast (Urban et al., 2007). In a nutrient-dependent manner, TORC1 phosphorylates several serines and threonines located in the C-terminus of Sch9 thereby controlling its activity. Activated Sch9 triggers different effects. First, TORC1-insensitive Sch9 alleles can partially protect cells from protein synthesis arrest (monitored by polysome profiles) and phosphorylation of eIF2α triggered by rapamycin. Second, Sch9 is able to phosphorylate Rps6 (the yeast ortholog of ribosomal protein S6) a component of the 40S small ribosomal subunit suggesting that indeed Sch9 is a genuine S6 kinase. Sch9 was also recently shown to control activation (rather than localization) of Maf1, an RNA Pol III repressor. In exponentially growing cells Sch9 is active and phosphorylates Maf1 on several serines leading to inactivation of Maf1. Rapamycin treatment causes inactivation of Sch9. Maf1, in turn, is dephosphorylated and represses RNA Pol III (Huber et al., 2009), which is in charge of translating the 5S rRNA and tRNA (Geiduschek and Kassavetis, 2001). These results demonstrate that some aspects of translation control by TORC1 in yeast are mediated by Sch9.

In yeast, TORC1 has also been shown to regulate translation initiation via eIF2 (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003). GTP-loaded eIF2 is required to deliver an initiator methionyl-tRNA to the 40S ribosomal subunit and eventually to the translation initiation codon of an mRNA. The α-subunit of eIF2 (eIF2α) is phosphorylated by Gcn2 during conditions where the pools of uncharged tRNAs are increased (starvation for instance) and this inhibits general

translation initiation (Sattlegger and Hinnebusch, 2005). On the other hand, phosphorylation of Gcn2 on serine 577 reduces the tRNA binding activity of Gcn2 and subsequently inhibits its kinase activity. Although the upstream kinase is unknown, phosphorylation of Ser-577 is promoted by TORC1: rapamycin treatment results in a rapid loss of Ser-577 phosphorylation, activation of Gcn2 kinase activity, increased eIF2α phosphorylation and ultimately decreased general translation initiation (Fig. 7).

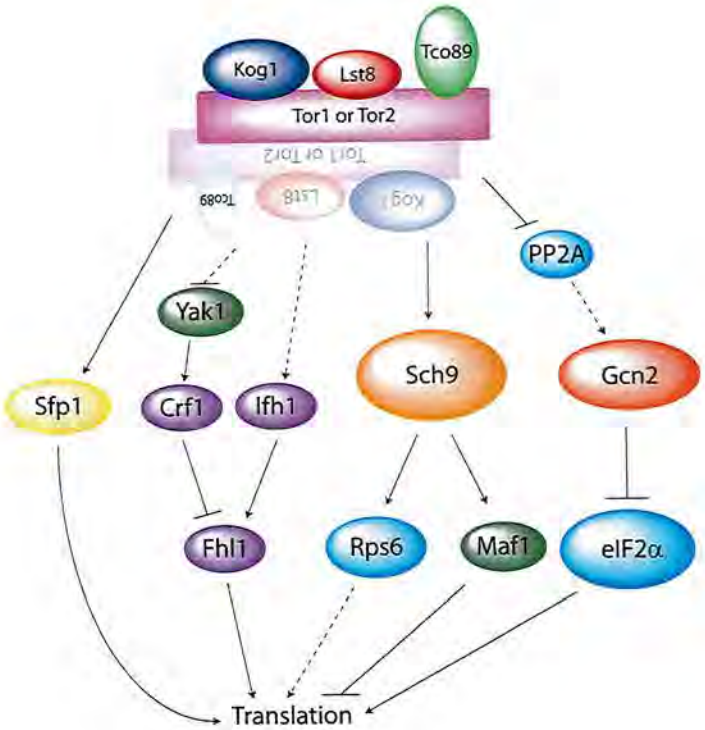

Fig. 7: TORC1 Controls Translation.
S. cerevisiae TOR complex 1, composed by either Tor1 or Tor2, Kog1, Lst8 and Tco89, controls translation via several mechanisms and effectors. TORC1 activates Sfp1, a transcription factor that controls expression of ribosome biogenesis (RiBi) genes, and Sch9, which phosphorylates the component of the small (40S) ribosomal subunit Rps6 and Maf1, an RNA Pol III repressor. TORC1 is also involved in the control of ribosomal protein (RP) genes via the Fhl1-Ifh1-Crf1 system and affects the activity of the Gcn2 kinase which inhibits eIF2α in response to uncharged tRNAs. Dotted lines are putative interactions.

5.6.1.2. Ribosome biogenesis

Rapamycin treatment, like nutrient limitation, triggers a dramatic inhibition of RNA Pol I and Pol III activity resulting in fast repression of RP, Ribi, rRNA and tRNA genes (Zaragoza et al., 1998). These regulatory aspects are dependent on proper function of TORC1 and are distinct from the branch of the pathway involved in translational initiation regulation (Powers and Walter, 1999). In particular, several transcription factors regulate RP gene expression in a TORC1-dependent fashion. Fhl1 is a forkhead DNA-binding protein localized to the promoters of most RP genes and is implicated in RNA Pol III function (Hermann-Le Denmat et al., 1994). Fhl1 binding to RP

promoters appears to be constitutive, and is facilitated by Hmo1, a high-mobility group protein, and Rap1, a protein required for expression of RP genes among many other activities (Hall et al., 2006). The mutually exclusive interaction of the *f*ork*h*ead-*a*ssociated (FHA) domain of Fhl1 with two other proteins, Ifh1 and Crf1, appears to be regulated by TORC1 activity (Martin et al., 2004). Ifh1-Fhl1 complexes stimulate, whereas Crf1-Fhl1 complexes suppress, expression of RP genes. Both Ifh1 and Crf1 are phosphoproteins with phosphorylation dictated by TORC1 activity (Fig. 7). Crf1 is phosphorylated directly by Yak1 (a protein kinase negatively regulated by TORC1 (Schmelzle et al., 2004)) and consequently translocates into the nucleus competing with Ifh1 for Fhl1 binding. How Yak1, or the yet unidentified Ifh1-kinase, is regulated by TORC1 is not entirely clear; possible upstream kinases include protein kinase A (PKA) and casein kinase II.

TOR-dependent regulation of RP genes is still observed in the absence of the Fhl1-Ifh1-Crf1 system, suggesting the existence of other mechanisms by which TOR regulates RP expression. One candidate is the transcription factor Sfp1 that interacts directly with TORC1 and is phosphorylated at multiple residues in a TORC1-dependent manner (Lempiainen et al., 2009) (Fig. 7). Furthermore, the Sfp1-interacting protein Mrs6, an essential Rab escort protein involved in intracellular vesicular trafficking (Benito-Moreno et al., 1994), regulates both Sfp1 nuclear localization and TORC1 activity, thereby indicating direct links between intracellular vesicular trafficking, TORC1 signalling, and ribosome biogenesis.

5.6.1.3. mRNA turnover

Not only production and utilization, but also stability and turnover of mRNA are crucial aspects for gene expression. mRNA decay begins with deadenylation, which is a process that ends with shortening of the poly(A) tail at the 3' end of the mRNA. Subsequently, deadenylated mRNAs can be either degraded in a 3' →5' direction by the cytoplasmic exosome, or decapped by Dcp1 and Dcp2 (Coller et al., 2001) and degraded in a 5' →3' direction by the Xrn1 exonuclease (Long and McNally, 2003). The first decay process is the most frequent in yeast and both nutrient limitation and rapamycin treatment accelerate this pathway, resulting in an enhanced turnover of some but not all mRNAs (Albig and Decker, 2001).

5.6.1.4. Permease sorting

S. cerevisiae can metabolize and utilize a wide variety of compounds for growth. As stated earlier, when yeast cells are growing on a mixture of different kinds of sugars, they will preferentially utilize glucose and fructose. A similar preference is observed for nitrogen sources: yeast normally

prefers fast and easily metabolizable nitrogen sources like ammonium, glutamine and glutamate over urea and proline (Magasanik and Kaiser, 2002). Yeast cells have a wide set of permeases with different substrate specificities, affinities and transport capacities. Expression, localization and activity of these transporters are nutrient-regulated (Magasanik and Kaiser, 2002) and TORC1 seems to play a very important part in those processes. Under optimal growth conditions, many high-affinity, selective permeases are expressed and targeted to the plasma membrane to uptake nutrients that are used directly in ATP production and/or protein synthesis. In contrast, in poor nutrient conditions, a few low-affinity, broad-specificity permeases are expressed and targeted to the plasma membrane to scavenge any organic compound usable to survive.

The idea that TORC1 is involved in permease activity arose from the observation that, like nutrient limitation or downshift of cells from good to poor nutrient conditions, rapamycin treatment quickly triggers internalization of some permeases from the plasma membrane to the vacuole for degradation (Schmelzle et al., 2004; Schmidt et al., 1998). The main player in the regulation of permeases is the kinase Npr1 whose phosphorylation state is controlled by TORC1. When TORC1 is inactivated, Npr1 is quickly dephosphorylated and activated. Via opposite and unknown mechanisms, Npr1 triggers internalization and degradation of the tryptophane permease Tat2 (Schmidt et al., 1998), while the general amino acid permease Gap1 is sorted to the plasma membrane (De Craene et al., 2001) (Fig. 8).

Although the mode of action of Npr1 has not been elucidated yet, the mechanisms of its regulation involve the Tor-controlled PP2A-like phosphatases Pph21, Pph22 and Sit4 with their associated subunit Tap42. When a preferred nitrogen source is present in the medium, Tor phosphorylates and thus activates Tap42 thereby promoting its binding and inhibition of the related phosphatases Pph21, Pph22 and Sit4. Upon inhibition of TORC1 by nitrogen starvation or addition of rapamycin, Tap42 is inhibited by the protein Tip41 (negatively regulated by TORC1) (Jacinto et al., 2001), and the phosphatases are now able to dephosphorylate their targets like Npr1.

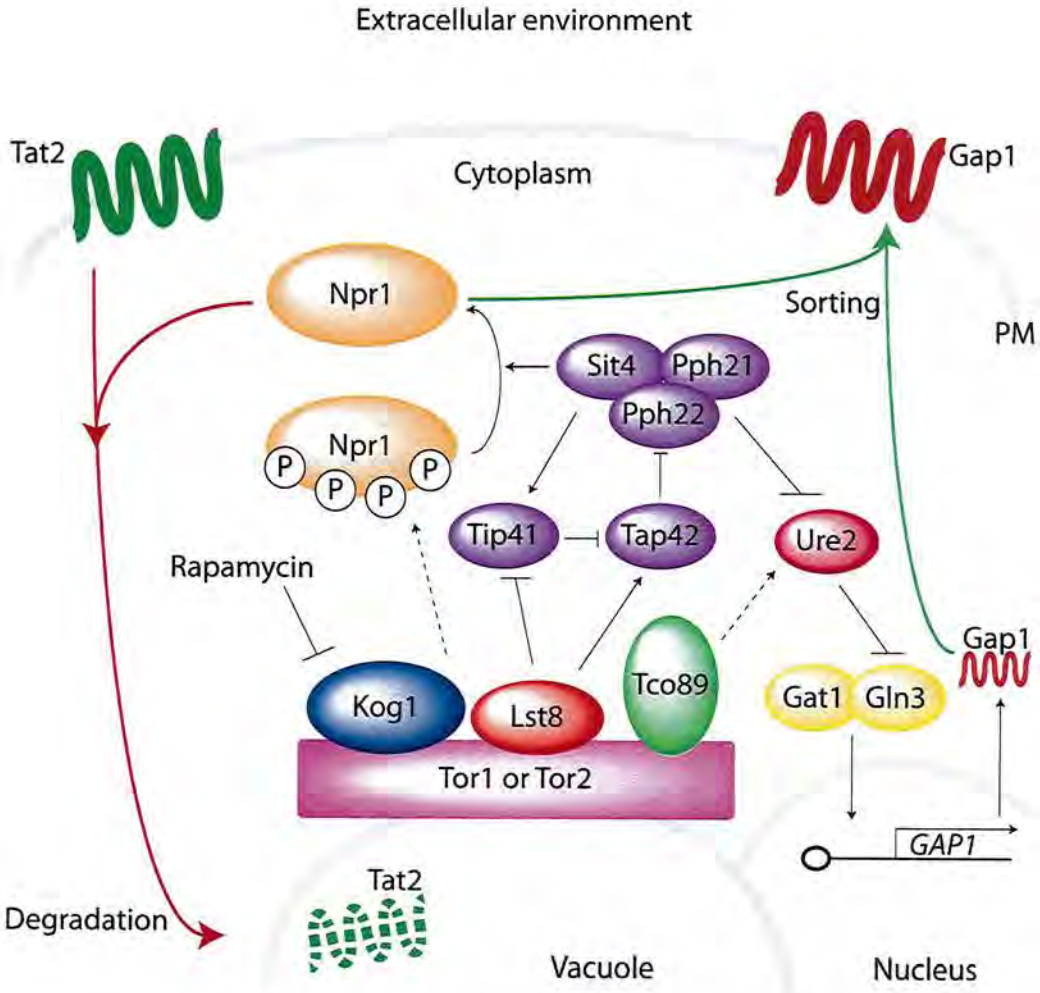

Fig. 8: TORC1 Controls the Localization of Amino Acid Permeases.
TORC1 pathway in yeast is responsible for localization of several permeases like Tat2 and Gap1. TORC1 controls sorting and degradation via the Npr1 kinase which, switched in the active form after rapamycin treatment or nutrient starvation, promotes Gap1 sorting to the plasma membrane and Tat2 internalization. *GAP1* is also a target of the NCR pathway whose main players are the transcription factors Gat1 and Gln3. Dotted lines represent putative or indirect interactions.

TORC1 controls permease activity also at the level of transcription via the GATA transcription factors Gat1 and Gln3. In normal growth conditions, Ure2 is activated by phosphorylation and sequesters the GATA transcription factors in the cytoplasm. Nitrogen starvation activates the PP2A-like phosphatases that, in this branch of the pathway, dephosphorylate the cytoplasmic repressor protein Ure2 and cause the dissociation and nuclear import (via Srp1 importin) of Gat1 and Gln3 where they can induce expression of the NCR-sensitive genes involved in import and catabolism of poor nitrogen sources (Beck and Hall, 1999) (Fig. 8).

5.6.1.5. Macroautophagy

Cellular activity is maintained by the continuous balance of synthesis and degradation of many proteins. Every protein has a defined lifetime, from a few minutes up to several days. The vacuole

(lysosome in higher eukaryotes) is the main intracellular compartment for degradation of long-lived protein. Since long-lived proteins account for more than 90% of total cellular protein, amino acids derived from digestion and degradation of these proteins may help cells to maintain the correct intracellular amino acids pool during starvation. Indeed, observation of cellular morphology during nitrogen starvation in vacuolar protease mutants, reveals appearance of a high number of small spherical bodies in the vacuole (Takeshige et al., 1992). Those spherical bodies are double-layered vesicles called autophagosomes, which enclose parts of the cytosol in their lumen and are degraded by vacuolar proteases via a process called macroautophagy. Upon fusion with the vacuole, the inner vesicle (autophagic body) is released inside where its membrane and its cytoplasmic cargo are degraded and eventually recycled (Yorimitsu and Klionsky, 2005). For example, macroautophagy is extremely important during sporulation which is normally triggered by nitrogen starvation. Cells can survive this differentiation process without any external supply of nitrogen, thanks to the bulk recycling of pre-existing, intracellular, proteins and nucleic acids.

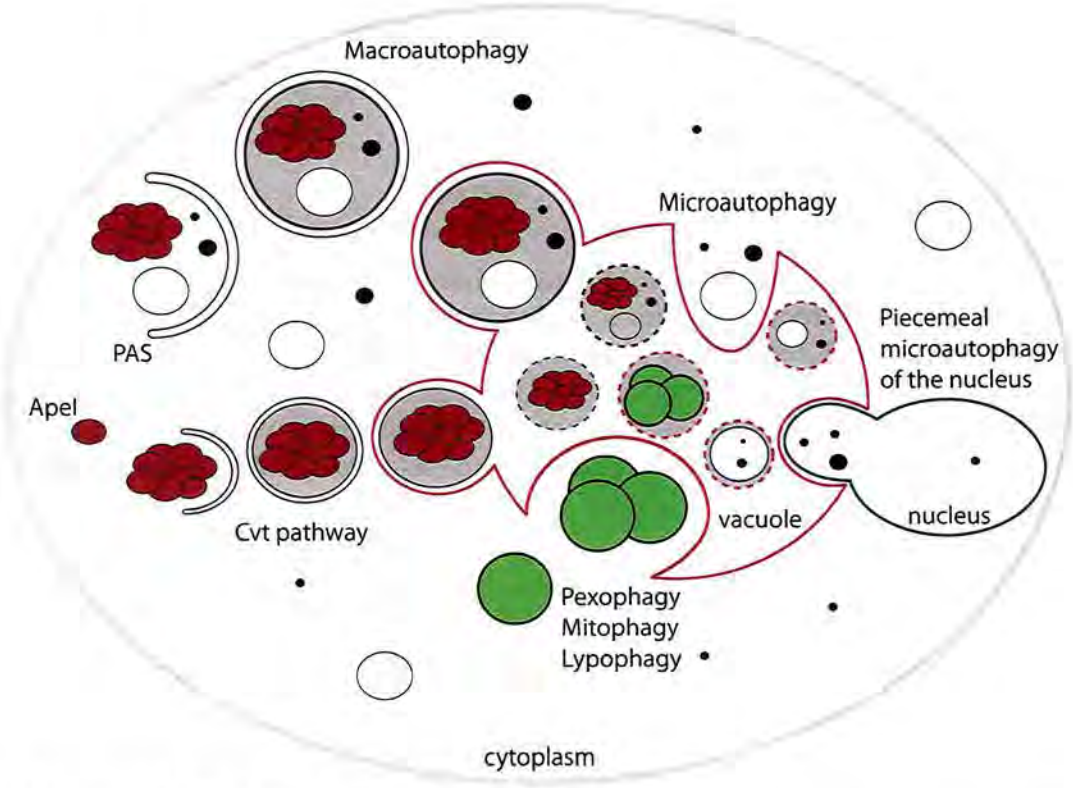

Fig. 9: The "Phagy" Family in *S. cerevisiae*.
During conditions of nutrient limitation, yeast is able to trigger catabolic processes to retrieve nutrients from degradation of dispensable cellular components. There are different kinds of autophagy, some are selective (pexophagy, lypophagy and mitophagy; cargoes in green), but normally macroautophagy is considered to be a non-selective process. The *c*ytoplasm to *v*acuole *t*argeting (Cvt) pathway is mechanistically similar to autophagy, but it is cargo selective (ApeI) and constitutive. The red organelle is the vacuole, the blue one is the nucleus.

Formation of autophagosomes can be easily monitored in yeast using the Atg8 protein fused with 3HA (Kirisako et al., 1999). Immunofluorescence analysis of subcellular distribution of Atg8 revealed a pattern of cytoplasmic dots under growing conditions. After few hours of starvation those dots were dramatically increased in number and size and completely filled the vacuolar lumen. Further analyses identified a novel pre-autophagosomal structure (PAS), in which Atg1, Atg2, Atg5, Atg8 and Atg16 (called in that study Apg1, Apg2, Apg5, Aut7 and Apg16 respectively) are assembled (Suzuki et al., 2001). The PAS is described as a flat membrane cistern that elongates and wraps itself around a portion of cytoplasm, forming a double-membrane bound autophagosome, that, since destined for destruction, must constantly be *de novo* created (Noda et al., 2000) (Fig. 9).

TORC1 inhibits catabolic processes and also regulates negatively macroautophagy. During growth on nutrient rich conditions, TORC1 regulates the interaction between Atg13 and the ser/thr kinase Atg1 via hyperphosphorylation of Atg13 (Funakoshi et al., 1997; Kamada et al., 2003). Macroautophagy under rich conditions is not completely abolished, but still flows at minimal levels. Upon nutrient starvation or rapamycin treatment, Atg13 is able to interact with and activate Atg1 which, in turn, recruits all the accessorial proteins to organize the PAS and increase the autophagocytic flux. Yeast cells with higher rates of macroautophagy show one typical abnormally big vacuole that, if the downstream events are somehow blocked (*e.g.* in a *pep4*Δ mutant), is full of small autophagosomal particles.

Macroautophagy has originally been considered as a non-selective process by which random portions of the cytoplasm are engulfed in the autophagosomes and sent to the vacuole for degradation. This is not always the case since cargo specificity has also been found in some cases. These include mitophagy (degradation of mitochondria (Kissova et al., 2004)), pexophagy (degradation of peroxisomes (Hutchins et al., 1999)) and lipophagy, which was discovered more recently (Singh et al., 2009) (Fig. 9). Finally, the constitutive *c*ytoplasm to *v*acuole *t*argeting (Cvt) pathway transports the resident hydrolase aminopeptidase I to the vacuole, using many of the same molecular components as autophagy (Khalfan and Klionsky, 2002).

5.6.1.6. Microautophagy and piecemeal microautophagy of the nucleus

Microautophagy is less well characterized than macroautophagy, but is also found in higher eukaryotes. Morphologically it consists in a tubular-like invagination of the vacuole following sequestration and degradation of a part of the cytoplasm (Yorimitsu and Klionsky, 2005). This process is extremely important to counterbalance the massive influx of membranous material

towards the vacuolar membrane caused by autophagy (Fig. 9). Thus, even though macroautophagy and microautophagy do not share any components, macroautophagy is considered as a prerequisite for the induction of microautophagy (Sattler and Mayer, 2000).

Little is known about microautophagy. This seems to be a process controlled to some extent by the EGO complex (see below), TORC1 (Dubouloz et al., 2005) and the *v*acuolar *t*ransporter *c*haperone (VTC) complex. Nitrogen starvation and rapamycin treatment have been shown to inhibit microautophay while the VTC complex is an important constituent of autophagic tubes and it is required for scission of microautophagic vesicles from these tubes (Uttenweiler et al., 2007).

Like macroautophagy, microautophagy can also be selective towards a specific organelle. At the moment, the only reported kind of selective microautophagy is called *p*iecemeal *m*icroautophagy of the *n*ucleus (PMN) (Fig. 9). This process occurs at specific junctions formed between the vacuolar protein Vac8 and its nuclear counterpart Nvj1. At these sites, the nucleus bulges into the vacuole and parts of the nucleus are sequestrated into invaginations of the vacuolar membrane that are pinched off into the lumen for subsequent degradation (Kvam and Goldfarb, 2004; Roberts et al., 2003). In constrast to simple microautophagy, PMN is highly induced upon rapamycin treatment. PMN was proposed to be biologically significant for the degradation of nuclear ribosomes that are no longer needed during starvation thus, TORC1 may control, via PMN, ribosome biogenesis (De Virgilio and Loewith, 2006; Roberts et al., 2003).

5.6.1.7. Control of entry into quiescence

Cells from organisms as diverse as bacteria and humans can enter a stable, non-proliferating quiescent state. Quiescent cells of eukaryotic and prokaryotic microorganisms can survive for long periods, sometimes even years, without nutrients. This alternative state of cells is still poorly understood, yet much benefit is to be gained by understanding it both scientifically and with reference to human health. For instance, understanding the processes that underline entry and exit from quiescence (or G_0) can be of crucial importance for the development of novel or supplementary immunosuppressants and anticancer therapies. It is also likely to provide significant insights into such diverse processes as aging and neurodegenerative diseases.

One important note is that quiescent cells are not dead; their metabolism is still active even though they do not proliferate, move or differentiate. Microorganisms can spread through the environment

as spores, blocked in this sleeping status and waiting to be spread in places where nutrient conditions are good and favourable for growth.

In yeast, quiescence is well characterized, but lots of aspects need to be still clarified. Quiescent yeast cells display numerous specific characteristics that differentiate them from proliferating cells: indeed, they do not proliferate and they are arrested in G_1; they fail to accumulate mass and volume; the overall transcription rate is three to five times lower than in logarithmic-phase cultures (Choder, 1991); they have a requirement for translation from internal initiation sites (internal ribosome entry site) (Paz et al., 1999); expression of a subset of genes is severely repressed (*e.g.* those encoding ribosomal proteins); expression of a subset of genes is strongly induced (Pedruzzi et al., 2003; Werner-Washburne et al., 1996); degradation of specific mRNAs is reduced at a stage preceding poly(A) shortening (Jona et al., 2000); overall protein synthesis is reduced (Fuge et al., 1994); chromosomes are condensed (Pinon, 1978); autophagy is induced to increase storage pools of nutrients (Noda and Ohsumi, 1998); cells develop thickened cell walls and are more resistant to digestion by zymolyase and to treatment with certain toxic drugs (de Nobel et al., 2000); and cells have increased thermotolerance and osmotolerance (Plesset et al., 1987).

To obtain a stationary phase culture of yeast in laboratory conditions is sufficient to grow in liquid a small amount of yeast for 5-7 days. When yeast is inoculated at a very low concentration, it will start growing exponentially until nutrients become limiting. At this stage, yeast cells arrest growth and enter in stationary phase as described above. Quiescence can be triggered more "artificially" using different methods: shifting a growing culture into a medium without a particular nutrient (nitrogen, glucose or others) or adding rapamycin, for instance.

Entry into quiescence in yeast is a completely reversible cycle. When glucose is added back to glucose-starved cells, they can easily start cycling again by activating or repressing a certain number of pathways. In general, the opinion that the lack of nutrients triggers entry into quiescence, while the resupply of nutrients triggers exit, is well supported by several lines of evidence.

Proper entry, suvival in, and exit from a quiescent state are key determinants of yeast life span. Three classes of mutants have been identified: the first class contains mutants that fail to enter properly into the quiescent state and thus die when starved. Mutants in the second class are also known as "starvation maintenance mutants", which successfully enter quiescence but cannot stay viable in that state. The third class includes mutants that are defective in exit from quiescence. They

can enter and survive properly stationary phase but are unable to return to the proliferating state when nutrients again become available (Gray et al., 2004).

As explained above many changes in the growth conditions like glucose starvation, nitrogen starvation, phosphate starvation, osmotic stress, heat shock and rapamycin addition drive cells into G_0 or a G_0-like state. The question that arises then is: are there many different molecular mechanisms that trigger entry into stationary phase or do most of the growth-regulating pathways converge on one common quiescence effector?

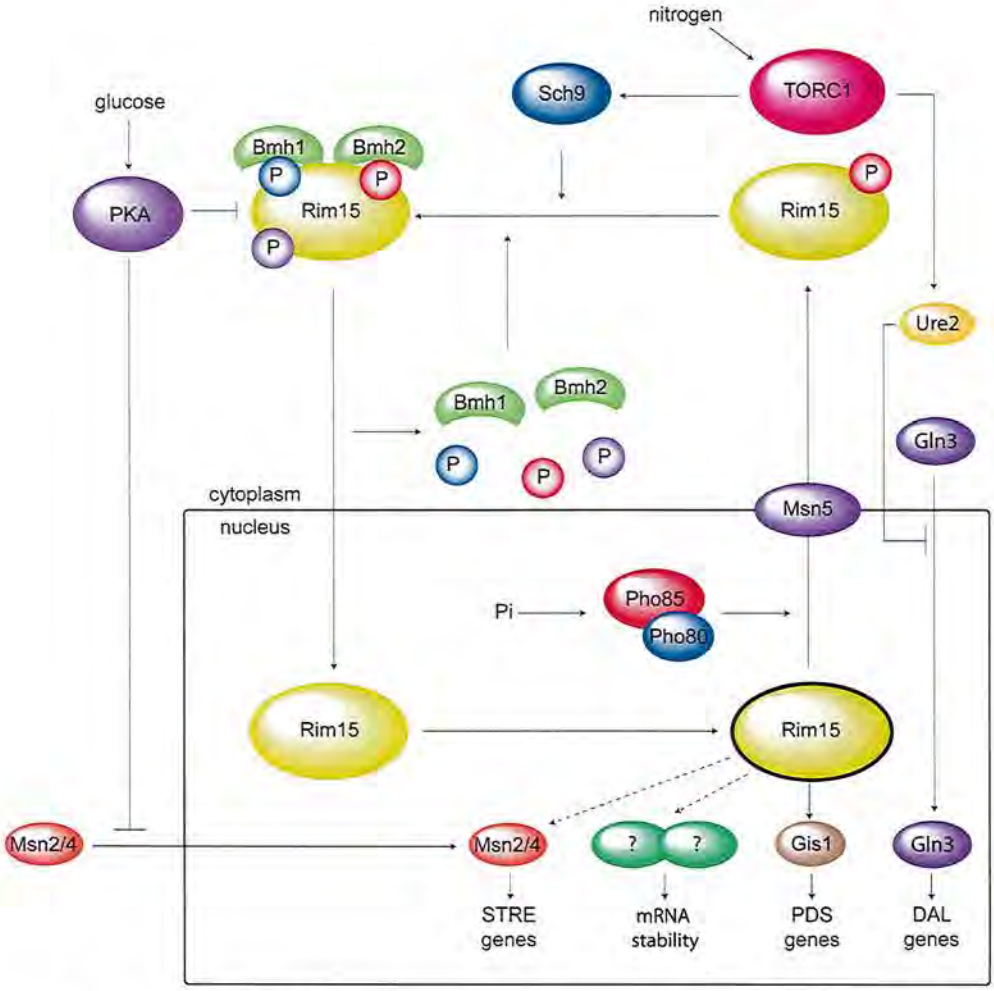

Fig. 10: Convergence of the Nutrient Sensing Pathways of Rim15, a Central Controller of Quiescence.
Nitrogen, phosphate and glucose are sensed respectively by the TORC1, Pho85 and PKA pathways. These pathways converge on the kinase Rim15, which is essential for proper start of the G_0 programme and controll its localization and activity. The mechanism by which Rim15 triggers entry into quiescence is not clear, but it seems that it facilitates transcription of quiescence genes and stabilizes mRNAs via Igo1 and Igo2. Black-framed Rim15 represents autophosphorylated Rim15, dotted lines are putative or indirect interactions.

The yeast Rim15 protein kinase was initially identified through a mutation that caused reduced ability to undergo meiosis (Vidan and Mitchell, 1997). As stated above, sporulation in yeast is part of a differentiation process by which diploid cells arrest growth (normally because of nitrogen starvation) and undergo meiosis to generate four haploid cells. If diploids cannot correctly arrest growth, they cannot subsequently undergo meiosis.

Rim15 was independently identified by another group as a potential binding partner of trehalose synthase Tps1. In this study, Rim15 was proposed for the first time to be an activator of stationary phase: indeed, loss of Rim15 was shown to cause defect in trehalose synthesis (Reinders et al., 1998) as well as many other phenotypes associated with inability to induce the G_0 program following glucose starvation. Biochemically, Rim15 activity was also shown to be PKA-regulated. During exponential growth on glucose, PKA inactivates Rim15 by phosphorylation on five different serine residues. After glucose starvation, and consequently inactivation of the PKA pathway, Rim15 is dephosphorylated and its activity increases. Moreover, Rim15 also undergoes an autophosporylation process (Vidan and Mitchell, 1997; Wanke et al., 2005) which is believed to stimulate its nuclear export (Wanke et al., 2005). On the other hand, PKA inhibition leads to the nuclear accumulation of the transcription factors Msn2, Msn4 that bind DNA and start the transcriptional G_0 program (Gorner et al., 1998; Pedruzzi et al., 2000; Pedruzzi et al., 2003) (Fig. 10).

Interestingly, rapamycin treatment of exponentially growing cells results in growth arrest that is associated with physiological changes, which are characteristic of stationary phase cells, like cell cycle arrest, repression of general transcription and mRNA translation and induction of stress response genes. Intriguingly, mutants that lack Rim15 fail to properly start the G_0 programme following rapamycin treatment (they fail to induce stationary phase genes and to accumulate reserve carbohydrates) indicating the possibility that Rim15 is downstream of both TOR and PKA. On the other hand, *rim15Δ* mutants still arrest growth following rapamycin treatment indicating that TOR controls growth rate via a Rim15-independent mechanism. The interaction between TOR and Rim15 have been further elucidated: inactivation of TORC1 by rapamycin results in dephosphorylation of the threonine 1075 in Rim15 which is critical for its binding to the cytoplasmic 14-3-3 anchor protein Bmh2 (Wanke et al., 2005). Thus, TORC1 and PKA signals converge on Rim15: TORC1 controls the localization and PKA the activity of Rim15 (Fig. 10).

TORC1 controls Rim15 also via the kinase Sch9. A recent study elucidated the involvement of Sch9 in preventing entry in stationary phase. During exponential growth, Sch9 interacts with and phosphorylates Rim15 on serine 1061 within a loop that is inserted between kinase subdomains VII and VIII. Mutation of this serine into alanine, which renders Rim15 not phosphorylatable anymore by Sch9, impaired cytoplasmic retention of Rim15 but does not affect its activity (since this is PKA-regulated process). Accordingly, phosphorylation of both serine 1061 and threonine 1075 in Rim15 may cooperatively mediate 14-3-3-protein-binding to guarantee optimal sequestration of Rim15 in the cytoplasm. Moreover, TORC1-insensitive alleles of Sch9, in which residues phosphorylated by TORC1 have been substituted with acidic amino acids (Urban et al., 2007), are able to partially prevent activation of Rim15 following rapamycin and caffeine treatment, but fail to do so in the presence of the Sch9-insensitive Rim15 allele (Wanke et al., 2008).

In addition to its role in glucose- and nitrogen-dependent signalling, Rim15 also controls entry into G_0 in response to a phosphate (Pi) starvation signal (Wanke et al., 2005). The mechanism by which the Pi signalling pathway controls Rim15 activity is pretty similar to the one elicited by TORC1: the complex Pho85-Pho80 phosphorylates Rim15 on threonine 1075 in the 14-3-3 binding site regulating its association with Bmh2. All the above data support a model in which Rim15 activity is regulated by at least four nutrient-regulated protein kinases namely PKA, TORC1, Sch9 and Pho85. Active nuclear Rim15 regulates entry into the stationary (G_0) phase via an unclear mechanism. It has been proposed that Rim15 could regulate chromatin remodelling in order to facilitate the interaction of the transcription factors Msn2, Msn4 and Gis1 with DNA. Msn2 and Msn4 regulate expression of stress responsive genes while Gis1 induces transcription of post-diauxic shift genes (Pedruzzi et al., 2000; Roosen et al., 2005) (Fig. 10). Active Rim15 also initiates an autophosphorylation process which stimulates its nuclear export via Msn5 (Wanke et al., 2005).

5.6.1.8. Lifespan regulation

Reduction of food intake, commonly referred to as dietary restriction (DR), has been shown to slow aging and extend lifespan in virtually every biological system examined (Longo and Finch, 2003). In yeast two different kinds of aging have been described: replicative aging (determined by the number of mitotic events a mother cell undergoes before senescence) and chronological aging (the time a nondividing population remains viable in liquid media) (Fabrizio et al., 2001; MacLean et al., 2001). Studies in yeast have demonstrated that impairment of conserved nutrient-responsive signal transduction pathways can phenocopy DR and extend both chronological lifespan (CLS) and replicative lifespan. Specifically, reducing the kinase activities of TORC1, the TORC1 substrate

Sch9 or PKA was found to extend CLS. On the other hand, any reduction of activity of the kinase Rim15 (the convergence point of different nutrient signalling pathways) can decrease CLS to some extent (Reinders et al., 1998; Wei et al., 2008). Moreover, dietary restriction fails to further increase CLS in cells lacking *TOR1* indicating that TOR is a target of DR in yeast (Kaeberlein et al., 2005). The working model by which TOR is modulated by nutrients (and thus by DR) and controls lifespan was further confirmed by another large-scale analysis which indicated TORC1 as a critical determinant of both replicative and chronological aging in yeast (Powers et al., 2006). Decreased activity of TOR and Sch9 orthologs increases life span also in higher organisms like *Caenorhabditis elegans* (Vellai et al., 2003) and *Drosophila melanogaster* (Kapahi et al., 2004), as does mutation of the TOR-regulated S6 kinase, which promotes ribosomal protein maturation in multicellular eukaryotes. This demonstrates that DR modulates lifespan through an evolutionary conserved mechanism. Two recent papers published by Harrison et al. (2009) and by Colman et al. (2009) reported increased longevity of mice fed with rapamycin and of rhesus monkeys undergoing caloric restriction, respectively. Thus, it is likely that the mechanism by which TORC1 controls longevity has been conserved over evolution.

5.6.2. Localization of TORC1

Several studies have already investigated the sub-cellular localization of TORC1 via localization of its subunits using different techniques like immunogold electron microscopy, GFP tagging, or indirect immunofluorescence (Araki et al., 2005; Cardenas and Heitman, 1995; Chen and Kaiser, 2003; Reinke et al., 2004; Sturgill et al., 2008). All these studies support the conclusion that TORC1 is associated with membranes, in particular with the vacuolar membrane, while TORC2 is located mostly to the plasma membrane (Kunz et al., 2000; Sturgill et al., 2008). The localization of TORC1 is particularly relevant, since the vacuole is thought to be the main nutrient reservoir containing especially large pools of basic amino acids.

The vacuole (or lysosome in higher eukaryotes) is a complex single layer membrane organelle; it is filled with water and contains inorganic and organic molecules (*e.g.* enzymes). The function of this organelle depends on the organism or cell type, but in yeast many vacuolar functions have been reported. The main function of the yeast vacuole is storage of nutrients obtained via uptake or via autophagy-dependent protein degradation (Mijaljica et al., 2007). The low vacuolar pH is critical because in the lumen many proteases that work at low pH are present, and it is maintained by the vacuolar membrane ATPase (V-ATPase) (Kane and Parra, 2000). The V-ATPase is a remarkably conserved protein complex that consumes ATP to generate a proton gradient across the vacuolar

membrane. It is composed of two different sectors that reversibly associate in a glucose-dependent manner to form a functional complex (Parra and Kane, 1998). The exact mechanism is still unclear but addition of glucose to cells growing on non fermentable carbon sources triggers a rapid association of the V-ATPase and subsequent acidification of the vacuolar lumen (Parra and Kane, 1998). Genetic deletion of V-ATPase subunits appears to be lethal in all organisms except fungi (Sun-Wada et al., 2000). Thus, because they are viable, yeast V-ATPase subunit deletion (*vma*$^-$) mutants provide invaluable insights into the functional importance of vacuolar acidification. Notably, one important feature of *vma*$^-$ mutants is that they are inviable on media buffered at neutral or high pH (Kane, 2006). This is in line with the observation that the same mutations confer lethality when combined with mutations that abolish endocytosis leading to the hypothesis that vacuolar acidification is an essential process also in yeast and that yeast can acidify the vacuole also via uptake of H^+ ions in the media via endocytosis (Munn and Riezman, 1994; Sambade et al., 2005).

Vma$^-$ mutants are also calcium-sensitive. This sensitivity results from the central role of the vacuolar Ca^{2+}/H^+ exchanger Vcx1 in calcium homeostasis and its dependence on a V-ATPase established H^+ gradient (Forster and Kane, 2000). Other metals are also sequestered in the vacuole, and the loss of V-ATPase activity is thought to compromise also the activity of some basic amino-acid antiporters (Ohsumi and Anraku, 1981; Sato et al., 1984). Basic amino-acids can accumulate to high levels in the vacuole, but there is little vacuolar storage of acid ones.

Another interesting function of the vacuole is its involvement in the response to osmotic stress. High extracellular salt concentrations trigger a very quick fragmentation of the vacuole in many small vacuoles. Indeed, mutants that lack structurally intact vacuoles are also osmosensitive reflecting a defect in osmoregulatory mechanisms (Banta et al., 1988).

Even if vacuoles can *de novo* originate in a yeast cell (Raymond et al., 1990), vacuolar growth and division must be coordinated with cell growth and division. Following movements of vacuoles *in vivo* it was possible to understand that the vacuole is first fragmented into smaller vacuoles (similarly to cells subjected to osmotic shock) that are subsequently translocated into the daughter cell (bud) via a tubular segregation structure. The newly formed cells contain several vacuoles that eventually fuse with each other to generate a bigger vacuole (LaGrassa and Ungermann, 2005). This process is called homotypic vacuolar fusion and it is also important for fusion of cellular membranes during secretion and endocytosis (Price et al., 2000). Homotypic fusion occurs in the

ordered stages of priming, docking and fusion. During priming, individual vacuoles are prepared for interaction with other vacuoles by assembling the soluble NSF attachment protein receptors (SNAREs) (Ungermann and Wickner, 1998). Activated t-SNAREs can interact with v-SNAREs of adjacent vacuoles leading to the docking step, during which the two vacuoles get for the first time in contact with each other. The interaction between SNARE proteins from apposed vacuoles becomes irreversible forming a trans-SNARE complex that triggers the release of lumenal calcium to interact with calmodulin and mediate downstream events that lead to fusion (Peters and Mayer, 1998). The HOPS complex (*ho*motypic fusion and vacuole *p*rotein *s*orting), composed of several proteins identified in a screen for vacuole morphology mutants (*vam* mutants) (Wada et al., 1992), is vital for the fusion event which is triggered, eventually, by the Vam6-regulated GTPase Ypt7 (Haas et al., 1995; Ungermann et al., 2000; Wurmser et al., 2000). The HOPS complex is composed of Vps18/Pep3, Vps11/Pep5, Vps33/Vam5, Vps16/Vam9, Vps39/Vam6 and Vps41/Vam2 and it was isolated using recombinant GST-Ypt7 that was pre-loaded with GTP and immobilized to Sepharose beads (Seals et al., 2000). Mutations in genes coding for HOPS complex subunits lead to a typical *vam*$^-$ phenotype. Interestingly, the HOPS complex (but not its target Ypt7) was also recently identified in a screen aimed to the identify synthetic lethal interactors with a *tor1*Δ mutant (Zurita-Martinez et al., 2007). Further analysis revealed a possible novel function of the HOPS complex as mediator of intracellular amino acid homeostasis for efficient TORC1 signaling. The still unanswered question is whether the HOPS complex influences TORC1 activity via controlling vacuolar morphology or via an unknown mechanism.

5.6.3. Upstream of mTORC1

The molecular mechanisms by which TOR proteins sense nutrient availability became clearer following the isolation of protein complexes associated with Tor1 and Tor2. Loewith *et al.* (2002) showed that TORC1 contains several accessorial subunits like Kog1 and Lst8. Both of them have mammalian counterparts, namely Raptor and mLst8 which are still able to bind to and form a complex with mTor1 (Hara et al., 2002). In yeast, both Kog1 and Lst8 are essential. Repression of Kog1 expression produces a phenotype that resembles the one of either TOR deficiency of rapamycin-treated cells. On the other hand, analysis of the downstream effects of specific mutations in Lst8 or deletion of *tco89* suggest also that those two subunits, like Kog1, act positively on TORC1 (Chen and Kaiser, 2003; Reinke et al., 2004). In yeast not much is known about the upstream events that regulate TORC1, but in mammals it has been shown that Raptor is necessary for binding to the downstream effectors of mTOR, S6K1, and 4E-BP1 (Nojima et al., 2003). On the other hand, mLst8 interacts specifically with the kinase domain of mTOR (independently of Raptor)

and plays a positive role in mTOR activation by nutrients stabilizing mTor association with Raptor (Kim et al., 2003).

mTOR, Raptor and mLst8 are components of a nutrient-sensitive mTOR complex 1 (mTORC1), it is still not clear how nutrients impinge on mTORC1. In higher eukaryotes, insulin and other growth factors dramatically increase the phosphorylation of S6K1 and 4E-BP1 in a rapamycin-sensitive manner. The phosphoinositide 3 kinase (PI3K) is considered to be the main actor in the nutrient-dependent regulation of mTORC1 for two main reasons: firstly, overexpression of an activated catalytic subunit of PI3K induces 4E-BP1 phosphorylation in the absence of growth factors or insulin (Gingras et al., 1998), secondly, overexpression of dominant-negative forms of p85 (the regulatory subunit of PI3K) inhibits insulin-induced phosphorylation of S6K1 (Ueki et al., 2000). On the other hand, the phosphatidylinositol-3 phosphatase PTEN (counteracting the activity of PI3K) regulates also phosphorylation levels of 4E-BP1: PTEN-deficient cells have high levels of 4E-BP1 and S6K1 phosphorylation (Neshat et al., 2001) (Fig. 11).

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), is a downstream effector of PI3K and has emerged as a critical mediator of mTORC1 activity. Prerequisite for Akt activation is the interaction between its pleckstrin homology (PH) domain and PIP3 causing subsequent translocation of Akt to the plasma membrane (Kandel and Hay, 1999). Full activation of Akt requires two independent phosphorylation events: the first one, on threonine 308, is mediated by the PIP3 dependent kinase Pdk1 while the second protein kinase, acting on threonine 473 is still controversial. Over time, various investigators have implicated several different classes of protein kinases in Pdk2 site phosphorylation (Delcommenne et al., 1998), whereas others suggest that autophosphorylation is the cause (Toker and Newton, 2000). More recently, it has been reported that members of the PIKK (phosphatidylinositol kinase-related kinase) family are responsible for Pdk2 site phosphorylation of Akt, including DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase) (Feng et al., 2004), the product of the gene mutated in ataxia telangiectasia (Viniegra et al., 2005), and the mTOR ortholog itself (Sarbassov et al., 2005). Overexpression of a dominant inactive form of Akt impairs insulin-mediated phosphorylation of 4E-BP1, thereby unequivocally implicating it as an upstream activator of mTORC1 (Gingras et al., 1998) (Fig. 11).

A major breakthrough in the understanding of how growth factors and Akt regulate mTORC1 activity was achieved by the observation that mutations in *Drosophila TSC1* or *TSC2* genes cause increased cell and organ size similar to that caused by mutation of dPTEN placing Tsc1 and Tsc2

proteins upstream of mTOR (Gao and Pan, 2001). Moreover, S6K1 is highly phosphorylated in mammalian cells lacking a functional Tsc1 or Tsc2 (Kwiatkowski et al., 2002) and Tsc2 itself is phosphorylated *in vivo* and inhibited by Akt (Inoki et al., 2002). In cells lacking Tsc2, S6K and 4E-BP1 are constitutively phosphorylated in a rapamycin-inhibitable manner, while overexpression of both (but not alone) Tsc1 and Tsc2 impairs insulin-stimulated phosphorylation of S6K1 and 4E-BP1 (Inoki et al., 2002). All those data support a model in which Akt controls mTor activity at least in part by inactivating Tsc2.

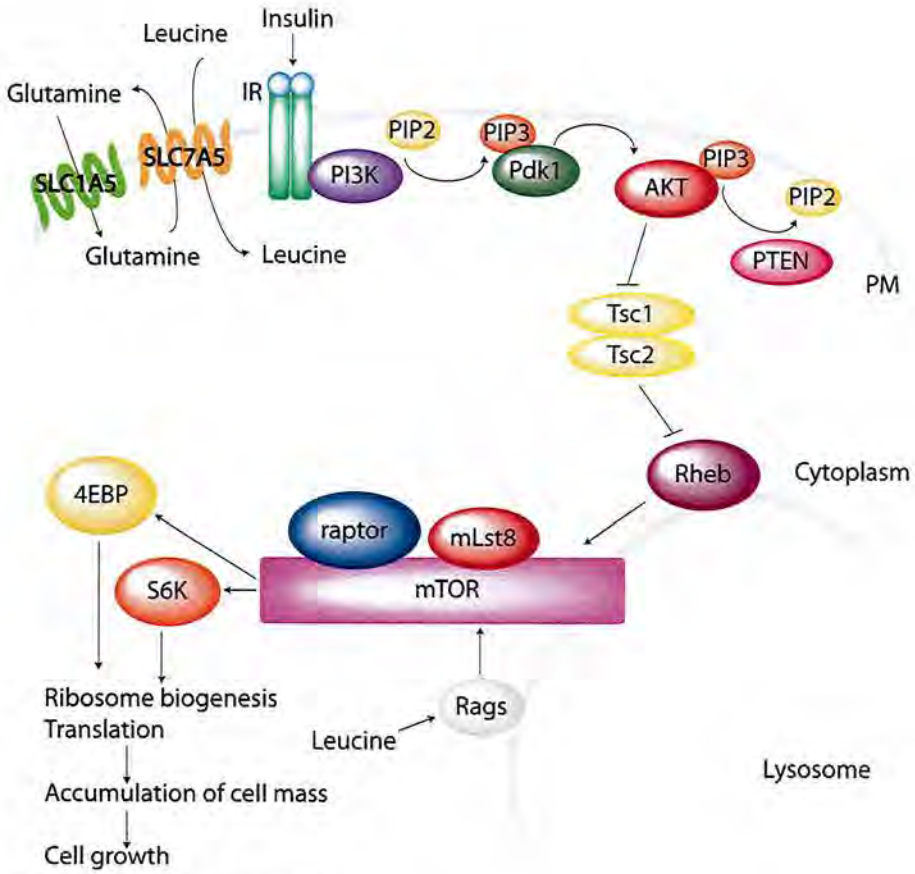

Fig. 11: Mammalian TORC1 Network.
Mammalian mTORC1 localizes to endomembranes (incluing late endosomes and lysosomal membrane) and responds to both amino acids and growth factors. While growth factors stimulate mTORC1 activity via Rheb, amino acids, especially leucine, activate via an unknown mechanism the Rag GTPases that are believed to control localization of mTORC1 towards Rheb-containing locations that are close to the lysosomal membrane.

The new question that arose at this point was: what is the biological activity of the heterodimer Tsc1-Tsc2? The N terminus of Tsc1 is homologous to the GAP of the small GTPase Rap. Indeed, in early studies it was reported that Tsc1 has a GTPase activity on the small G-proteins Rap1 and Rab5 (Wienecke et al., 1995). Subsequently, a genome-wide screen for proteins involved in cell growth control in *Drosophila* uncovered the small GTPase Rheb (Im et al., 2002), which acts

downstream of Akt as a positive regulator of mTor (Saucedo et al., 2003; Zhang et al., 2003). Further analysis completed the model: Tsc2 binds to and shows activity towards Rheb indicating a crucial involvement of this protein in the Akt-mTORC1 pathway (Castro et al., 2003). Finally, overexpression of Rheb in mammalian cells caused hyperactivation of mTORC1 even in the absence of growth factor or in the presence of wortmannin (PI3K inhibitor) and furthermore, overexpression of a dominant-negative form of Rheb blocks activation of mTOR by growth factors and insulin (Tabancay et al., 2003). These results demonstrate that Rheb is a positive upstream regulator of mTORC1 that acts downstream of the Tsc1-Tsc2 dimer (Fig. 11).

A notable exception is the amino acid supply, as the mTORC1 pathway remains sensitive to amino acid starvation (but not to insulin or growth factors withdrawal) even in the absence of Tsc1-Tsc2 proteins (Smith et al., 2005). The first candidates shown to interact with mTOR in an amino acid-dependent manner were the four small G-proteins of the mammalian Rag family (RagA, RagB, RagC and RagD). The RagA and RagB proteins are very similar to each other and are orthologous to yeast Gtr1, while the RagC and RagD are similar and orthologous to Gtr2. They interact with the mTORC1 subunit raptor always as heterodimers (RagA-C or RagB-C, but not RagA-B for instance) in which RagA-B are preferentially loaded with GTP while RagC-D with GDP. The interaction of the Rags with mTORC1, probably as an effect of their nucleotide-bound state, is sensitive to amino acids and in particular to leucine. Rag heterodimers in which RagB is GTP-locked are constitutively bound to mTORC1 also in the absence of leucine. As a consequence, mTORC1 is constitutively activated, as judged from *in vivo* phosphorylation of S6K1, also during amino acid starvation (Sancak et al., 2008). However, unlike Rheb (Sancak et al., 2007), the mammalian Rags were shown not to stimulate *in vitro* the kinase activity of mTORC1. On the other hand, it has been proposed that the Rag proteins in mammals control the localization of mTORC1 to the late endosomal and lysosomal compartments in response to amino acids to be properly activated by Rheb. This would explain why activators of Rheb, like insulin, do not stimulate the mTORC1 pathway when cells are deprived of amino acids and why Rheb is necessary for amino acid–dependent mTORC1 activation. An independent study completed recently in *Drosophila* gave similar results leading to the conclusion that Rag proteins act in parallel with, or upstream of, Rheb to stimulate cell growth in response to amino acid via control of localization of TORC1 (Kim et al., 2008) (Fig. 11).

Shortly after the recent discovery of the mechanism that controls mTORC1 in response to amino acids it has also been clarified how amino acids, glutamine in particular, exert their functions in

regulation of mTORC1. Cellular uptake of glutamine is the rate limiting step for essential amino acids (EAA) and growth factor-regulation of mTORC1. Following uptake via the solute carrier family 1 member 5 (SLC1A5), glutamine is effluxed within a few minutes, leading to reciprocal uptake of leucine mediated by SLC7A5 and rapid activation of S6K1 presumably via Rags (Nicklin et al., 2009) (Fig. 11). Even though mTORC1 has been shown in several studies to be an integrator of different growth signals (amino acids, insulin, growth factors, PA, ATP, etc...), the mechanism by which leucine is sensed and is transmitted to the Rags needs to be further elucidated.

5.6.4. Upstream of yeast TORC1

PIP3 seems to have no reported role in the physiology of yeast *Saccharomyces cerevisiae* (Wera et al., 2001). Nonetheless, the yeast genome encodes all the proteins that might be involved in a control mechanism of TORC1 via a pathway equivalent to the mammalian PI3K/PTEN/Akt pathway. Yeast has two functional PDK1 orthologs (Pkh1 and Pkh2) involved in cell integrity and endocytosis (Roelants et al., 2002), an apparent PTEN orthologue (Tep1) of uncharacterized biological function (Heymont et al., 2000) and an Akt-like protein kinase (Sch9), which however lacks an evident PH domain and acts downstream of TORC1 to control ribosome biogenesis, lifespan and cell-size (Jorgensen et al., 2004; Urban et al., 2007). So far neither of these proteins, nor the yeast Rheb ortholog Rhb1, have been shown to be part of the signaling network upstream to TORC1. In particular, Rhb1 function in yeast appears only to be necessary for uptake of arginine (Urano et al., 2000).

The first proteins candidate to be upstream of yeast TORC1 were identified in a genome-wide screen for mutants unable to re-grow after a rapamycin-mediated growth arrest. This screen led to the identification of the EGO (from *e*xit from rapamycin induced *g*rowth arrest) complex, composed of the previously uncharacterized vacuolar-localized proteins Ego1 and Ego3 and small RagC/D homolog Gtr2. The data presented were favouring a model in which the EGOC was necessary for activation of microautophagy, a process necessary to counterbalance the massive membrane influx to the vacuole caused by rapamycin treatment. Moreover, the activity of the EGOC was shown to be mainly dependent on the nucleotide bound state of Gtr2, where alleles mimicking the GTP loading status of Gtr2 were inhibitory versus microautophagy (Dubouloz et al., 2005). EGOC function becomes essential for growth when TORC1 activity is reduced. This places the EGOC either upstream of (working with other unidentified pathways in TORC1 regulation), or in parallel of TORC1.

The EGOC was independently identified by another group in a screen aimed to identify genes essential for the translocation of the general amino acid permease Gap1 to the plasma membrane. Gap1 is a low affinity permease and its activity is reported to be controlled mainly by the nitrogen source in the medium (Courchesne and Magasanik, 1983). Transcription of *GAP1* mRNA is controlled by the TOR pathway-dependent transcription factor Gln3. Thus, Gap1 is expressed in cells grown on urea, proline or glutamate, but not in cells grown on glutamine as a nitrogen source (Stanbrough and Magasanik, 1995). Post-translational control of Gap1 localization underline a similar regulatory mechanism: in poor nitrogen sources Gap1 is sorted to the plasma membrane and stabilized there by the protein kinase Npr1 (De Craene et al., 2001), while re-addition of glutamine or ammonium leads to internalization of Gap1 and delivery to the vacuole with subsequent proteasome-dependent degradation (Hein et al., 1995). For those reasons, *gap1*Δ mutants, as well as mutants unable to sort Gap1 to the plasma membrane when requested, should be able to grow properly on media that contains the proline toxic analogue L-azetidine-2-carboxylic acid (ADCB). Using a random transposon-insertion-generated library, Kaiser and colleagues identified mutants showing increased resistance to ADCB suggesting that they are unable to sort Gap1 to the plasma membrane. Among the genes identified in this collection there were the previously characterized genes *EGO1*, *EGO3*, *GTR2* (Dubouloz et al., 2005), *GTR1* (Schurmann et al., 1995) and *LTV1* (Loar et al., 2004), which interact with each other in the GSE (*G*ap1 *s*orting in the *e*ndosome) complex. All GSE complex components are necessary to exert its function in Gap1 sorting; moreover, the activity of the complex was reported to be mainly dependent on the nucleotide loading status of Gtr1 where Gtr1-GTP is activatory (Gao and Kaiser, 2006).

Curiously, the positive role of the GSE complex in Gap1 sorting was observed in cells grown on ammonium, a condition under which most wild-type cells repress *GAP1* transcription and sort Gap1 to the vacuole. Based on these observations, several questions arise:

(i) Is Gap1 sorting different only in the strain background used by the Kaiser's group?
(ii) Is it possible that the EGOC regulates TORC1 indirectly by controlling Gap1 sorting?
(iii) May the EGOC control yeast TORC1 similarly as Rag GTPases control mTORC1?

- Question (i) is addressed in Chapter I. The experiments presented in the work from Gao and Kaiser were repeated using two different yeast genetic backgrounds (the BY4741 and the KT1960). Secondly, I show that involvement of the EGO/GSE complex in Gap1 sorting is dependent on the yeast background used.

- Question (ii) will be also addressed in Chapter I in which I test a possible involvement of Gap1 in TORC1 signalling. The results exclude the possibility that the EGOC regulates TORC1 indirectly by controlling Gap1 sorting. Moreover, I show that in the absence of Gap1 the activity of TORC1 is not affected during growth in both rich- and poor-nitrogen media.
- Nevertheless, question (iii) remains the main topic of Chapter I. Given the non-reproducibility of the Gap1 results in the BY4741 background, the second part of Chapter 1 is more dedicated in the confirmation of the hypothesis presented in Dubouloz et al. (2005): is the EGOC a direct activator of TORC1? I show that the function of the EGOC is amino acid-dependent activation of TORC1 via Gtr1, whose nucleotide-binding status is dictated by the GEF Vam6.

The results presented in Chapter I will lead to another fascinating question: is the EGOC activating TORC1 in response to cytoplasmic or vacuolar pools of amino acids or both? This question will be addressed in Chapter II where will be speculated that TORC1 is a sensor of intracellular amino acids, with the EGOC that mainly mediates the signal in response to cytoplasmic amino acid availability.

Different genome-wide screenings have been achieved in order to find unknown additional regulators of the EGOC and of TORC1. The Chapter III will be dedicated to present the complete set of the results obtained. The most interesting candidates will be discussed.

Finally, all the conclusions and outline will be summarized in Chapter IV, dedicated to a general discussion.

6. Chapter I:

6.1. The Vam6 GEF controls TORC1 by activating the EGO complex

Binda M, Péli-Gulli MP, Bonfils G, Panchaud N, Urban J, Sturgill TW, Loewith R, De Virgilio C.

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Own contributions to this Chapter:

- **Fig. 1**: EGOC Acts Upstream of TORC1;
- **Fig. 2B**: EGOC Does Not Control TORC1 via Gap1 Sorting;
- **Fig. 3A**: Alternative Nucleotide-Bound States of Gtr1 and Gtr2 Are Required for Amino-Acid-Dependent Control of TORC1;
- **Fig. 4A, B, D, E**: Gtr1, Preferentially in Its GTP-Bound Form, Interacts Physically with TORC1;
- **Fig. 5A, E, G, I, H, J**: Vam6 Is a Gtr1 Nucleotide Exchange Factor;
- **Fig. 6B**: TORC1 and EGO Complex Subunits Colocalize with Vam6 at Vacuolar and Endosomal Membranes;
- **S1**: Loss of the TORC1 Downstream Effector Gln3 Suppresses the Defect in Recovery following Rapamycin Treatment, but Not the TORC1 Activity Defect, of EGO Complex Mutants;
- Strains and plasmids listed in Supplemental Data I;
- Supplemental Data II.

6. Chapter I:

6.2. Supplemental Data I

6. Chapter I:

6.3. Supplemental Data II

6.3.1. Introduction

In the previous section it was shown that the EGOC controls TORC1 activity in response to leucine. The C-terminal fragment of Sch9, direct target of TORC1 (Urban et al., 2007), has been used as readout to test the activation state of TORC1 in *egoc* mutants. The results clearly showed that, in the absence of EGOC subunits, Sch9 phosphorylation is affected. However, other readouts of TORC1 are known. Maf1, for instance, was recently shown to be directly phosphorylated by Sch9 in a TORC1 dependent manner (Huber et al., 2009). In exponentially growing cells, Sch9 is kept by TORC1 in its active state and phosphorylates Maf1. This phosphorylation event is inhibitory: Maf1 is kept in its inactive form in the cytoplasm where it cannot repress RNA Pol III (Towpik et al., 2008). In turn, when cells are treated with rapamycin, Sch9 is dephosphorylated and Maf1 is activated, enters the nucleus and represses RNA Pol III. Thus, the phosphorylation state of Maf1 can also be used as readout for TORC1 activity.

Additionally, Npr1 is a protein kinase that can be used as readout for TORC1 activity. The exact role of Npr1 is not clear, but it has been shown to be involved in several aspects of permease sorting in a TORC1-dependent manner. When yeast cells are actively growing, Npr1 is kept in its inactive phosphorylated state. When cells are treated with rapamycin, Npr1 is quickly dephosphorylated and triggers internalization of the tryptophane permease Tat2 from the plasma membrane for degradation (Schmidt et al., 1998). On the other hand, Npr1 was also proposed to sort Gap1 from the Golgi to the plasma membrane during growth on poor nitrogen sources (De Craene et al., 2001). Npr1 dephosphorylation upon rapamycin treatment is controlled by Tap42 but, however, it is not clear if it can also be directly phosphorylated by TORC1 (Schmidt et al., 1998).

In this section, Maf1 and Npr1 phosphorylation status will be analyzed in *egoc* mutants. The aim of these experiments is to test if inactivation of TORC1 caused loss of EGOC affects also the function of Maf1 and Npr1.

6.3.2. Maf1 is correctly phosphorylated in *egoc* and *torc1* mutants

Since TORC1 activity is dramatically reduced upon loss of EGOC and TORC1 subunits (see Chapter I), Maf1 phosphorylation levels were also determined in *egoc* and *torc1* mutants. For this purpose, the corresponding mutants were transformed with a centromeric plasmid expressing endogenous levels of HA-tagged Maf1, and TCA-precipitates were analyzed by western blot using anti-HA antibodies. Maf1 is normally phosphorylated in all exponentially growing and CHX-treated mutants. On the other hand, it is rapidly dephosphorylated upon rapamycin or caffeine treatment

(Fig. 1). Thus, in contrast to the results obtained for Sch9 (section 6.1., fig. 1A), Maf1 seems not to be affected by loss of EGOC subunits. To explain this result it should be considered firstly that phosphorylation of Maf1 might take place in the nucleus, thus it might respond to an EGOC-independent TORC1 pool. In line with this hypothesis there is the fact that TORC1 has also been localized in the nucleus (Li et al., 2006). Secondly, Maf1 is not directly phosphorylated by TORC1, but by its downstream effector Sch9. It is worth noting that dephosphorylation of Sch9 in the *egoc* mutants is not complete (Chapter I, Fig.1A). Since the total pools of Sch9 are analyzed in that assay, it is not possible to exclude the existence of an EGOC-independent pool of Sch9 that is localized in the nucleus and that controls activity of Maf1. If this hypothesis is correct it should be noted that this nuclear pool of TORC1 is insensitive to CHX and, as a consequence, probably, also to amino acids.

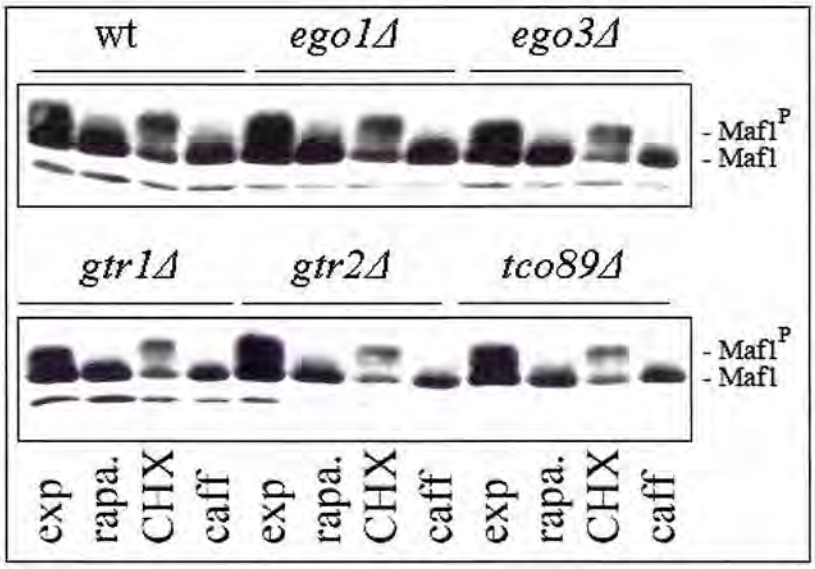

Fig. 1: Maf1 is Correctly Phosphorylated in *egoc* and *torc1* Mutants.
egoc and *torc1* mutants were transformed with a plasmid expressing Maf1-HA from its endogenous promoter. Cells were grown exponentially in YPD and treated with vehicle (exp), 200ng/ml rapamycin (rapa), 25µg/ml cycloheximide (CHX) or 20mM caffeine (caff) for 30 minutes. Cell extracts were subjected to SDS-PAGE, and immunoblots were probed with anti-HA antibodies.

6.3.3. Npr1 stability is affected in the *egoc* mutants

TORC1 was reported to control the activity and localization of different permeases via one of its effectors, the protein kinase Npr1 (De Craene et al., 2001; Schmidt et al., 1998). The functionality of Npr1 depends on the nitrogen source: good nitrogen sources promote its inactivation, whereas poor nitrogen sources, nitrogen starvation, or rapamycin treatment lead to its activation. Its mode of action is not clear yet, but Npr1 is reported to stabilize Gap1 at the plasma membrane and,

reciprocally, negatively regulates specific amino acid transporters like the tryptophan permease Tat2. Since Npr1 phosphorylation is considered to be a TORC1 readout, Npr1 phosphorylation levels were tested in *egoc* and *torc1* mutants. For this purpose, these mutants were transformed with a centromeric plasmid expressing endogenous levels of HA-tagged Npr1, and TCA-precipitates were analyzed by western blot using anti-HA antibodies. The results, however, are difficoult to interpret: in *egoc* and *torc1* mutants, Npr1 steady-state levels were dramatically reduced especially in the *tco89*Δ strain in which Npr1 is undetectable. Interpretation of the phosphorylation state of Npr1 is thereby difficult, however it seems that in exponentially growing *egoc* mutants, Npr1 is less phosphorylated with respect to the wild-type (Fig. 2).

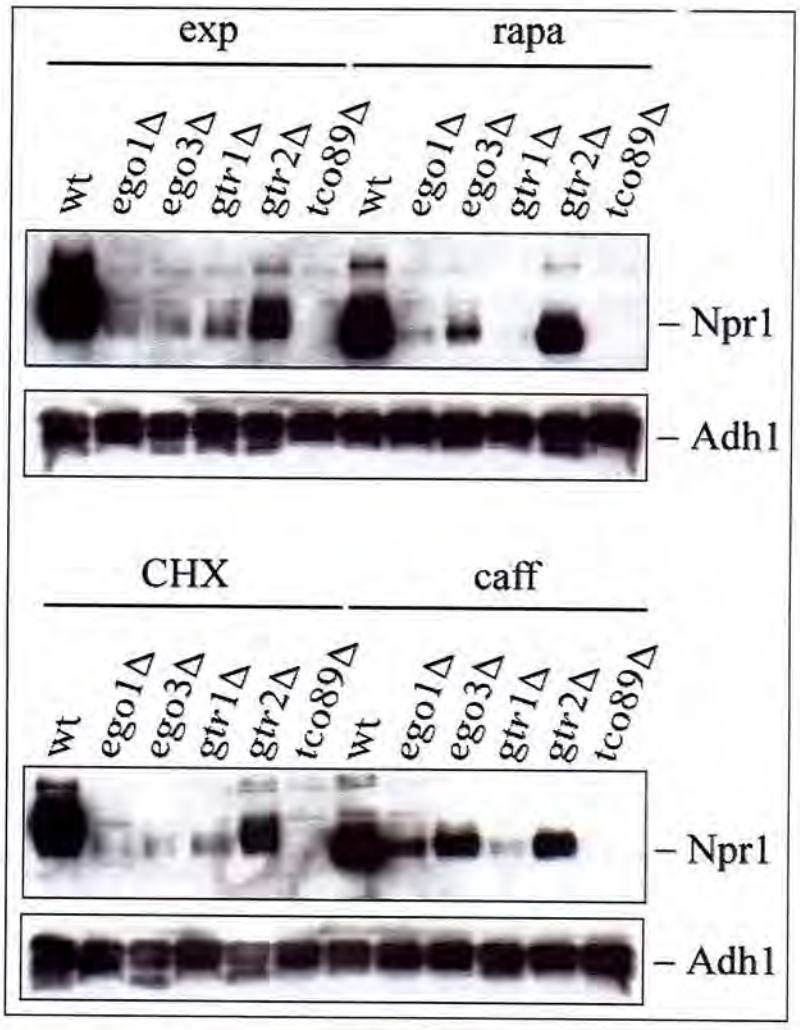

Fig. 2: *egoc* and *torc1* Mutants Display Lower TORC1 Activity.
egoc and *torc1* mutants were transformed with a plasmid expressing Npr1-HA from its endogenous promoter. Cells were grown exponentially in YPD and treated with vehicle (exp), 200ng/ml rapamycin (rapa), 25µg/ml cycloheximide (CHX) or 20mM caffeine (caff) for 30 minutes. Cell extracts were subjected to SDS-PAGE, and immunoblots were probed with anti-HA antibodies or anti-Adh1 antibodies for loading control.

It is unclear if in the *egoc* mutants Npr1 levels are reduced because Npr1 is less expressed or less stable. The fact that Npr1 contains PEST sequences that render it particularly unstable in conditions of reduced protein synthesis (Vandenbol et al., 1990) like, for instance, when TORC1 activity is reduced, may favour the latter hypothesis. This would be in agreement with EGOC functioning as upstream regulator of TORC1.

6.3.4. Tat2 stability is not affected in the *egoc* mutants

The Npr1 kinase, via a still unknown mechanism, inhibits tryptophan import probably by increasing the rates of Tat2 degradation. In line with this interpretation, it has been shown that rapamycin treatment, like nitrogen or amino acid starvation, or Npr1 overexpression, leads to a rapid degradation of Tat2 (Beck and Hall, 1999). In the previous section it was shown that the steady-state levels of Npr1 are altered in the *egoc* mutants and it was not possible to clearly assess the phosphorylation status of Npr1 in the mutants. However, Npr1 seemed to be dephosphorylated in exponentially growing *egoc* mutants, which would predict that in those strains the permease Tat2 is not stabilized at the plasma membrane. To test this hypothesis Tat2 expression levels were tested in those mutants. As shown in Fig. 3, in both BY4741 (A) and KT1960 (B) wild-type and various *egoc* and *torc1* mutants, Tat2 is correctly expressed under normal growth conditions and disappears completely after one hour of rapamycin treatment (200ng/ml). Additionally, proper activity of Tat2 at the plasma membrane was assayed by growing the mutants in the KT1960 background (which is auxotrophic for tryptophan) on media with very low doses of tryptophan (Umebayashi and Nakano, 2003). Under these conditions, Tat2 is essential for tryptophan uptake. In line with the previous experiments, none of the mutants seemed to be particularly affected for growth on a low concentration of tryptophan. This indicates that the lack of the EGOC does not cause mislocalization of Tat2 (Fig. 3C).

If Npr1 is really dephosphorylated in the *egoc* mutants (Fig. 2), the observation that Tat2 is not affected in the same mutants might be contradictory with the literature since active Npr1 is expected to favour degradation of Tat2 (Schmidt et al., 1998). However, it should be remark that in *egoc* mutants, the steady-state levels of Npr1 are dramatically reduced. Thus, the amounts of Npr1 left in yeast cells might not be enough to trigger a complete degradation effect on Tat2.

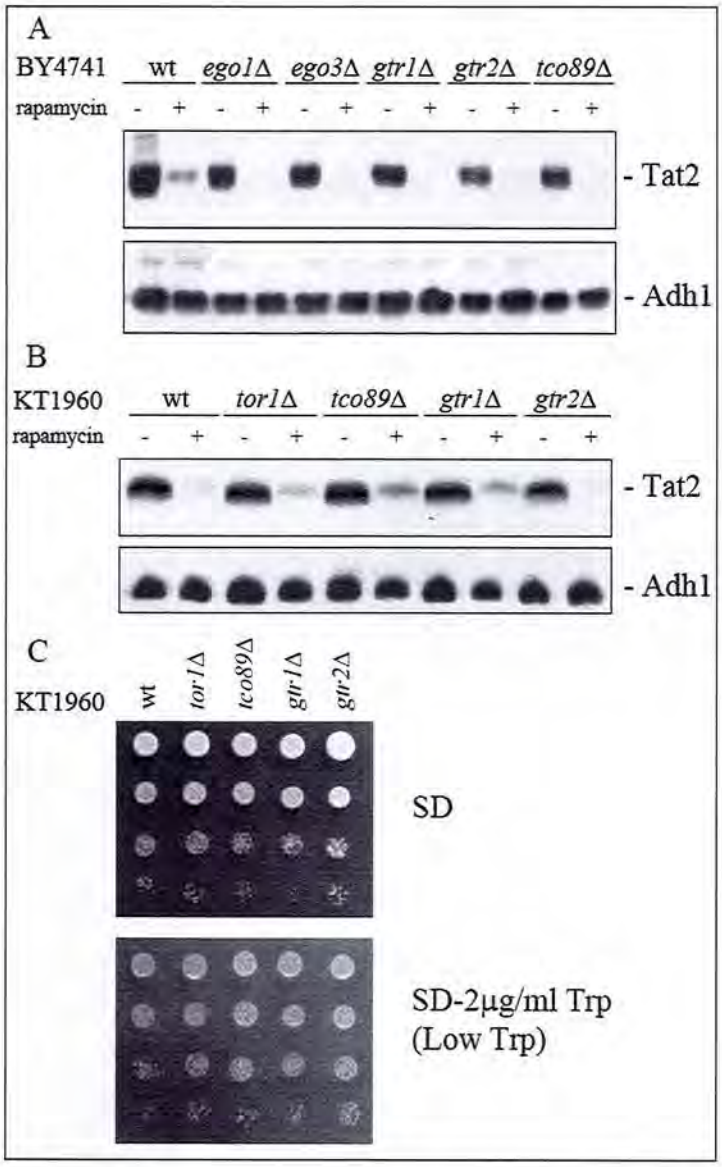

Fig. 3: The Tryptophan Permease Tat2 is Correctly Stabilized and Active in Both *egoc* and *torc1* Mutants.
(A-B) *egoc* and *torc1* mutants in the BY4741 background (A) and KT1960 background (B) were transformed with a plasmid expressing Tat2-HA from its endogenous promoter. Cells were grown exponentially in YPD and treated with vehicle (exp) or 200ng/ml rapamycin (rapa) to induce Tat2 degradation. Cell extracts were subjected to SDS-PAGE, and immunoblots were probed with anti-HA antibodies or anti-Adh1 antibodies for loading control.
(C) KT1960 background *egoc* and *torc1* mutants auxotrophic for *trp1* were grown exponentially in SD medium, harvested, serially diluted and spotted on SD-complete medium or SD medium containing only 2µg/ml tryptophan to check Tat2 activity. Cells were grown for 2 days at 30°C.

6.3.5. Gap1 is correctly sorted to the plasma membrane in *egoc* mutants in the KT1960 background

Previously in this Chapter, it was shown that the EGOC is not involved in the Gap1 sorting process in the BY4741 background (Binda et al., 2009). To further confirm these results, the effects on the

localization and activity of Gap1 were also tested in *egoc* and *torc1* mutants in the KT1960 background. To this end Gap1 localization was analyzed with both microscopy and fractionation assays (like in Chapter 1) in cells growing in synthethic medium containing 10 mM proline. In line with the results obtained in the BY4741 background, subcellular distribution of Gap1-GFP exhibited, in both wild-type and *egoc* and *torc1* mutants, a typical localization pattern: Gap1 is sorted to the plasma membrane when cells are growing in proline medium and internalized for degradation upon addition of ammonium (Fig. 4A).

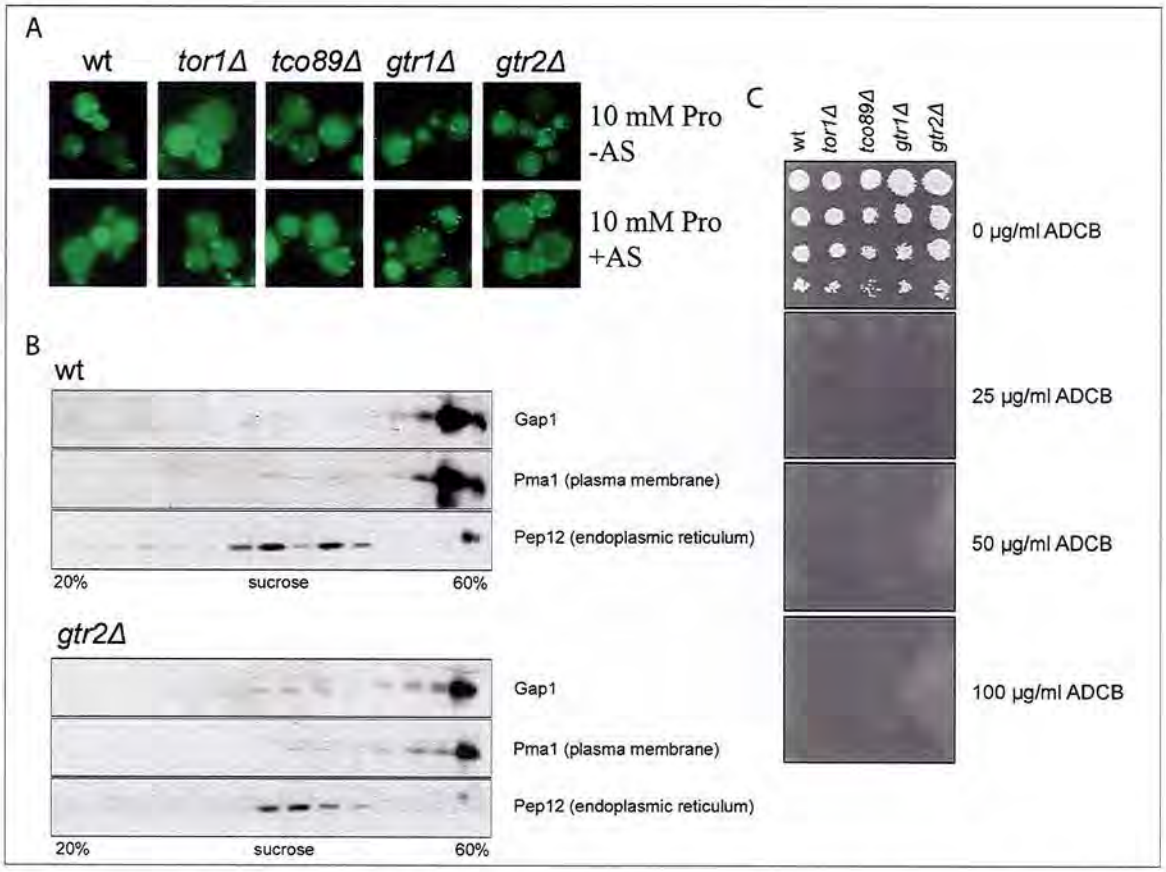

Fig. 4: EGOC Does Not Control Gap1 Localization in the KT1960 Background.
(A) *egoc* and *torc1* prototroph mutants expressing a plasmid-encoded Gap1-GFP under the control of the galactose-inducible *GAL1* promoter were grown in 1% raffinose, 2% galactose medium containing 10mM proline as sole nitrogen source and analyzed by fluorescence microscopy before (-AS) and after (+AS) addition of 5mg/ml of ammonium sulphate (AS).
(B) Prototrophic wild-type and *gtr2Δ* mutants growing on SD medium containing proline as sole nitrogen source were lysed and fractionated by equilibrium density centrifugation on continuous 20%-60% sucrose gradients (Kaiser et al., 2002). The fractions were assayed for the presence of Gap1, the plasma membrane protein marker Pma1, and the endosomal marker protein Pep12 using specific antibodies.
(C) Prototrophic wild-type and mutants were spotted on medium containing 10mM proline as sole nitrogen source and different concentrations of the proline toxic analogue ADCB and grown for 2 days at 30°C.

In line with this experiment, subcellular fractionation analysis showed that Gap1 was always present in Pma1-positive fractions when either wild-type or *gtr2Δ* cells were grown in proline containing medium (Fig. 4B). The activity of Gap1 at the plasma membrane was additionally tested by growing cells on different concentrations of the toxic proline analogue L-*a*zeti*d*ine-2-*c*ar*b*oxylic acid (ADCB) (Fig. 4C). Under poor nitrogen conditions, ADCB is primarily taken up by Gap1, with only a minor contribution of Put4 (Hoshikawa et al., 2003). Wild-type, *egoc* and *torc1* mutants were equally sensitive to ADCB when grown on proline. Taken together, these results show that mutations in EGOC and TORC1 do not affect sorting of Gap1 to the plasma membrane during growth on proline as sole nitrogen source in both BY4741 and KT1960 yeast backgrounds.

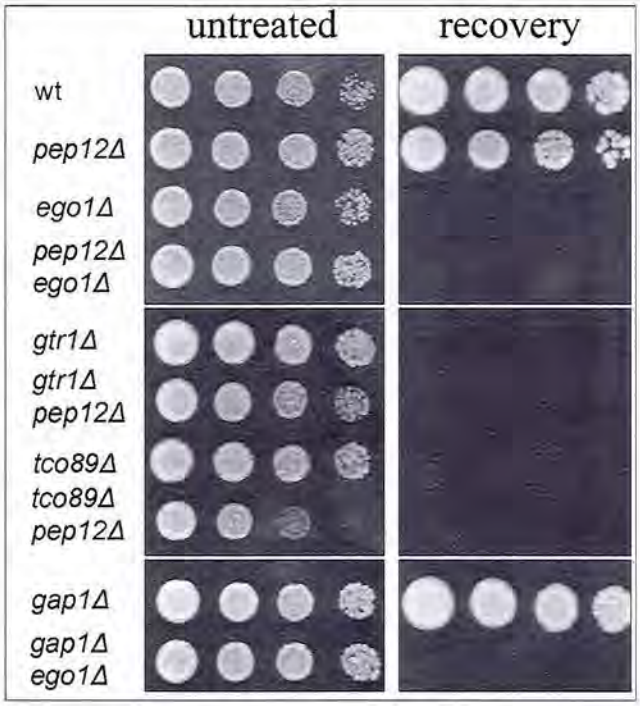

Fig. 5: Deletion of *PEP12* Does Not Suppress the *ego* Phenotype of *ego1Δ* Mutants in the BY4741 Background.
The indicated strains were grown exponentially in YPD, treated for 6 hours with rapamycin (200ng/ml), then washed twice and spotted as 10-fold serial dilutions on YPD plates. Cells were grown for 2 days at 30°C.

In their study, Gao and Kaiser showed that deletion of *PEP12* suppresses the Gap1 sorting defect of *gtr1Δ* cells. Accordingly, they propose that deletion of *PEP12* prevents newly made Gap1 from entering the endosome-to-vacuole traffic route, and directly sorts it to the plasma membrane. Accordingly, they concluded that Gtr1 may be required for Gap1 delivery to the plasma membrane, probably by retrieving it from the late endosome to the plasma membrane via a novel pathway. We therefore tested whether loss of Pep12 may suppress the defect of *egoc* and *torc1* mutants to recover from a rapamycin block. Moreover, it was also tested the possibility that a mislocalization of Gap1

in *egoc* mutants could be the cause of the *ego* phenotype. To do so, deletion of *GAP1* was combined with deletion of *EGO1* and this mutant was tested for its ability to recover from a rapamycin block. Neither loss of Pep12, nor loss of Gap1, did suppress the *ego* phenotype (Fig. 5). In all, these results lead to the conclusion that the EGOC has no role in Gap1 sorting process in the BY4741 background. Interestingly, *pep12Δ* mutants show a slight synthetic growth defect when combined with *tco89Δ* mutants suggesting that probably the endosome-to-vacuole traffic route is important in conditions of low TORC1 activity.

6.3.6. Gtr1 does not bind directly to Tco89 and Tor1

Sabatini and colleagues reported that mammalian Rags bind to raptor (Sancak et al., 2008), the mammalian ortholog of Kog1. This result is quite surprising because some genetic experiments presented in the previous chapter were favouring a model in which the EGOC is controlling TORC1 via Tco89, a homolog of which apparently does not exist in mammals. On the other hand, hyperactive alleles of Tor1 cannot suppress the low TORC1 activity phenotype of a *tco89Δ* strain, indicating that Tco89 is also an essential subunit for proper TORC1 activity. The interaction between Gtr1 and TORC1 was tested in *tco89Δ* and *tor1Δ* strains using the same biochemical assay described in the previous chapter. While in *tco89Δ* cells Kog1-TAP seems to be less stable (or less expressed), deletion of *TOR1* does not exert the same effect. However, GST-Gtr1 co-precipitates with Kog1-TAP in the same way in wild-type and both *tco89*Δ and *tor1*Δ strains, indicating that neither Tco89 nor Tor1 are essential for Gtr1 to bind TORC1 (Fig. 6).

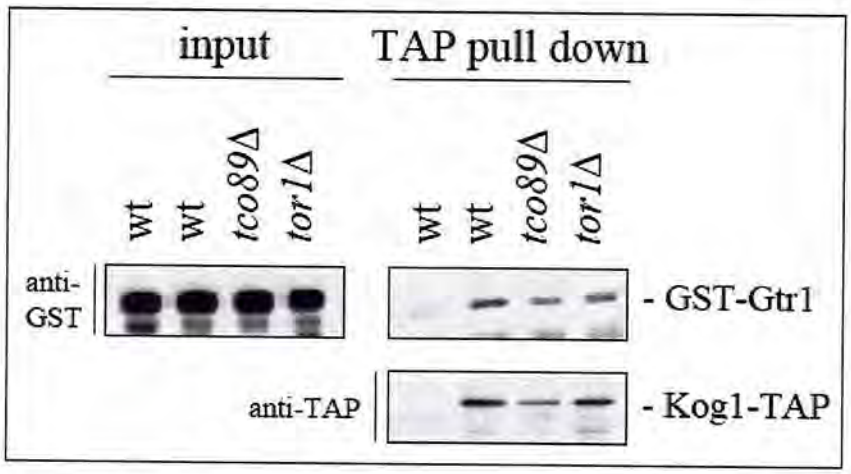

Fig. 6: Gtr1 Associates with TORC1 even in the Absence of Tor1 or Tco89.
Wild type control cells (left) or strains expressing Kog1-TAP were transformed with plasmids expressing Gst-Gtr1. Cell lysates (input) and TAP pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

6.3.7. Conclusions

The EGOC is a vacuolar-localized complex that was recently shown to control TORC1 activity in response to amino acids. Different readouts of TORC1 like phosphorylation state of Maf1 and Npr1 have been analyzed in *egoc* mutants, and the results obtained favour a speculative model in which the EGOC does not affect the functions of TORC1 in control of Gap1 sorting (De Craene et al., 2001; Schmidt et al., 1998) or in control of ribosome biogenesis (Huber et al., 2009).

The role of the EGO/GSE complex in Gap1 sorting proposed by Gao and Kaiser (2006) was additionally tested in the KT1696 background. In line with the results previously obtained with the BY4741 background, also in the KT1960 background Gap1 sorting at the plasma membrane during growth on poor nitroge sources is not affected in the *egoc* mutants. An explanation of this discrepancy was given previously: the SYHO yeast strain used by the group of Kaiser was shown to carry a mutation at the *PER1* locus which is linked to the *GDH1* gene encoding a glutamate-dehydrogenase (Courchesne and Magasanik, 1983). Thus, it would be really interesting in the future to test the effects of mutations in *GDH1* on TORC1 pathway and on Gap1 sorting.

7. Chapter II:

TORC1 activation: vacuolar vs cytoplasmic amino acid signal

7.1. Introduction

Amino acids and nutrients in the growth environment dictate production, localization and activity of most of the yeast permeases. Some amino acids need to be properly sensed by specific receptors located on the surface of the cells, others are sensed indirectly during their metabolism. In this context, one central amino acid sensing system is the *S*sy1-*P*tr3-*S*sy5 (SPS) sensor. Ssy1, Ptr3 and Ssy5 are components of a plasma membrane system (SPS sensor) that functions in the first step of a signalling cascade involved in sensing the presence of extracellular amino acids (Forsberg and Ljungdahl, 2001; Klasson et al., 1999). The cascade is activated by amino acid binding to Ssy1, which transmits the signal intracellularly through Ssy5 and Ptr3. Ssy5 is a protease that activates by cleavage the two transcription factors Stp1 and Stp2 thereby allowing them to enter the nucleus and activate the expression of target genes encoding the plasma membrane amino acid permeases Gnp1 and Apg1 (Liu et al., 2008). Yck1 and Yck2 are two kinases that are probably recruited by Ssy1 and that phosphorylate Ptr3 on several sites for full activation (Spielewoy et al., 2004). Inactivation of signalling in the absence of external amino acids is mediated by the Rts1/PP2A phosphatase complex, which was proposed to be a TORC1-controlled mechanism (Eckert-Boulet et al., 2006). Moreover, TORC1 has been also recently reported to control the degradation of the transcription factor Stp1. Addition of rapamycin triggers rapid disappearance from the nucleus and Sit4-dependent degradation of Stp1-GFP. On the other hand, expression levels of Stp1 affect cells sensitivity towards rapamycin. It has been postulated that higher levels of Stp1 trigger higher uptake of amino acids and a consequently a boost in TORC1 activity (Shin et al., 2009). Interestingly, external leucine is reported to be the most important amino acid for activation of the SPS sensor (Gaber et al., 2003; Liu et al., 2008).

As stated above, amino acids control also localization of other permeases. Tat2 for instance is sorted to the plasma membrane when tryptophan levels are low (Umebayashi and Nakano, 2003) and internalized for degradation in case of nitrogen starvation or during growth on high tryptophan concentrations. Even though the exact mechanism is not known, it may be possible that external tryptophan concentrations are sensed by specific receptors that generate a signal in order to drive changes in the metabolism and sorting of the permeases.

Once taken up, intracellular amino acids can have different fates depending on growth conditions. Amino acids can be stored in the vacuole, utilized in the cytoplasm as building blocks for protein synthesis, used in mitochondria for energy production or can be metabolized further to produce

other essential compounds. For those reasons, transport of amino acids between the vacuole and the cytoplasm must be an efficiently regulated process. Basic amino acids, like asparagine and lysine, are mainly stored in the acidic vacuolar lumen. The other amino acids are distributed between the cytoplasm and the vacuole and are transported back and forth by the *a*mino acid *v*acuolar *t*ransporters (AVT), proteins localized at the vacuolar membrane (Russnak et al., 2001). In particular Avt3 and Avt4 have been described to counteract the action of the importer Avt1. They export glutamine, leucine, isoleucine and tyrosine from the vacuolar lumen back to the cytoplasm. Avt6, on the other hand, is thought to export acid compounds like glutamate and aspartate. The exact mechanism is still unknown, but apparently transport of those amino acids across the vacuolar membrane needs a proton gradient. Deletion of the three exporters is expected to result in the increased vacuolar accumulation of key amino acids critical for TORC1 activity. Another class of proteins was recently identified to be involved in transporting basic amino acids into yeast vacuoles. Those proteins are called *v*acuolar *b*asic *a*mino acid (VBA) transporters and represent the main route for uptake of basic amino acids into the vacuole. In particular, a *vba1*Δ *vba2*Δ *vba3*Δ triple mutant fails to properly store basic amino acids in the vacuole (Shimazu et al., 2005). It is not clear however, whether the VBA proteins act as proton antiporters.

It is not known up to know how the bi-directional transport of amino acids across the vacuolar membrane is controlled. Notably, in a recent phosphoproteomic screen, the amino acid transporter Avt1 was isolated as putative target of TORC1 network (Huber at al., 2009) highlighting the possibility that TORC1 controls the intracellular amino acid permeases dictating their activity accordingly to cellular needs. Nevertheless, this hypothesis has not been tested yet.

Active transport is not the only way by which amino acids are transported into the vacuole. As explained in Section 5.7.5. (Fig. 8), autophagy is a catabolic process involving the degradation of proteins or cellular components in the vacuole. It is negatively regulated by nutrients (especially nitrogen) via the TORC1 pathway (Kamada et al., 2003). Inhibition of TORC1 via nitrogen starvation or rapamycin addition triggers a rapid increase in the autophagocytic flux towards the vacuole resulting in lumenal accumulation of amino acids that are subsequently exported to the cytoplasm for utilization (Yang and Klionsky, 2007).

It is extremely important for cells to continuously sense the external supply as well as the internal levels of amino acids. As stated above, different amino acid receptors like the SPS sensor are located on the cell surface to continuously monitor the external nutrient conditions. On the other

hand, TORC1 seems to be a good intracellular sensor for nutrients for several reasons: i) it is apparently activated by nutrients (leucine, glutamine, glucose) (Binda et al., 2009; Dubouloz et al., 2005; Crespo et al., 2002); ii) it controls localization and activity of some permeases like Gap1 and Tat2 (De Craene et al., 2001; Schmidt et al., 1998); iii) it coordinates protein synthesis rates and cell growth (Lempiainen et al., 2009) and iv) it is mainly localized at the vacuolar membrane (Sturgill et al., 2008). For those reasons it is reasonable to think that TORC1 responds to different amino acid pools in order to coordinate cell growth with nutrient and amino acid availability.

Given the recent discoveries of the EGOC as an upstream activator of TORC1, the aim of this chapter is to further study several aspects of the amino acid sensing mechanisms. In particular, it will be investigated which amino acid pools impinge on TORC1 and if the EGOC is important to mediate a cytoplasmic rather than a vacuolar amino acid signal, if not both.

7.2. Vacuolar amino acid pools may be important for TORC1 signalling

Since TORC1 was proposed to be controlled by glutamine and/or leucine levels (Binda et al., 2009; Dubouloz et al., 2005), the effects of the triple deletion *avt3Δ avt4Δ avt6Δ* mutant were tested on TORC1 activity. The idea is that upon deletion of those three genes, cells accumulate more amino acids like leucine and glutamine in the vacuole thereby resulting in higher TORC1 activity. As expected, triple mutant showed increased resistance towards rapamycin as shown by their faster growth on a medium containing a low rapamycin concentration of 10 ng/ml (Fig. 1A). This result indicates that vacuolar amino acid pools might have an effect on TORC1 signalling. Interestingly, the same mutations can partially suppress the *ego* phenotype of *ego3Δ* and *gtr1Δ* strains showing that the vacuolar amino acid signal might impinge on TORC1 in an EGOC-independent manner (Fig. 7B-C). These results confirm the existence of a growth control mechanism originating at the vacuolar membrane and pinpoint the amino acids glutamine and leucine as key metabolites in TORC1 signalling.

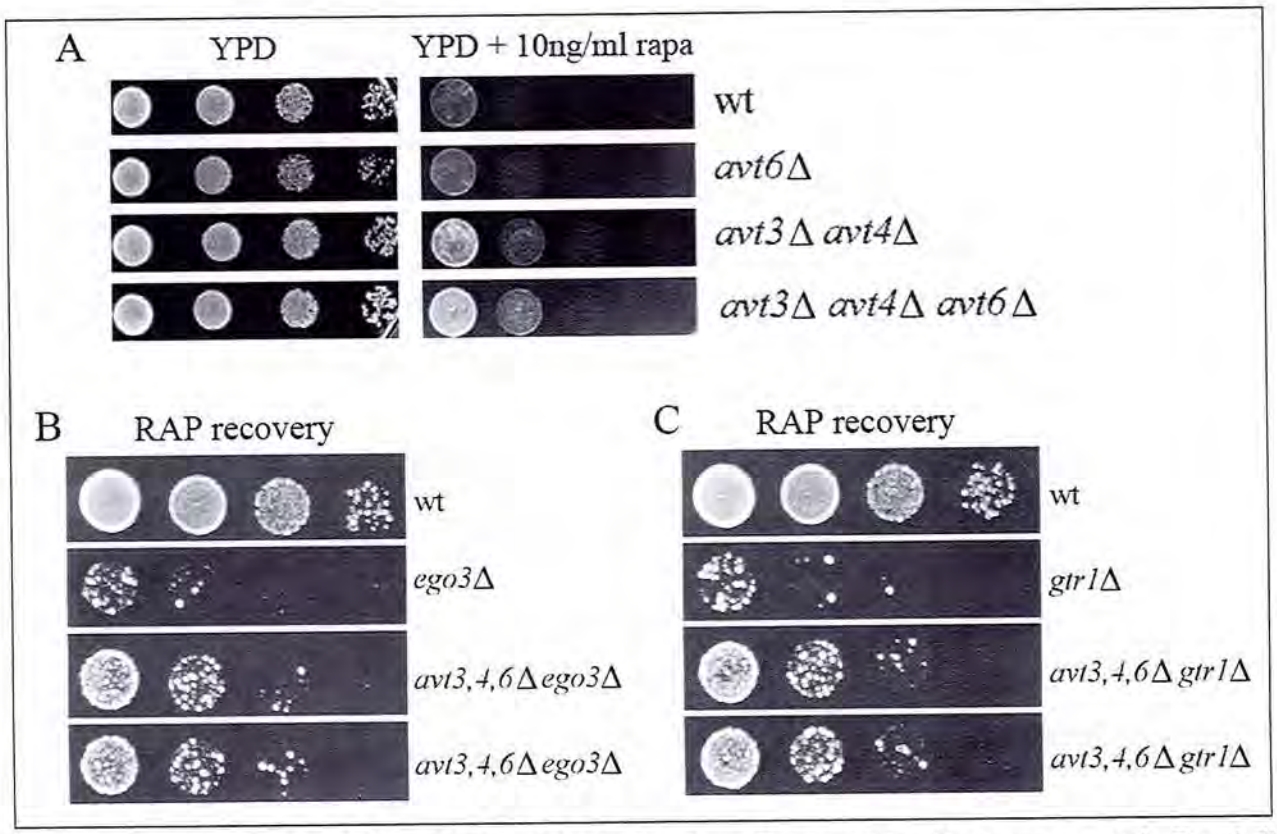

Fig. 1: Deletion of *AVT3, AVT4, AVT6* Confers Rapamycin Resistance and Partially Suppresses the *ego* Phenotype of *ego3Δ* and *gtr1Δ* Mutants.
(A) The indicated mutants were exponentially grown on YPD, serially diluted and spotted on YPD and YPD containing 10ng/ml rapamycin. Cells were grown for 36 hours at 30° C.
(B-C) The indicated mutants were exponentially grown in YPD, treated for 6 hours with rapamycin (200 ng/ml), then washed twice and spotted as 10-fold serial dilutions on YPD plates. Cells were grown for 2 days at 30° C.

7.3. The V-ATPase may control TORC1 via regulation of vacuolar amino acid pools

Considering the amino acid storage function of the vacuole, it is possible that inactivation of the V-ATPase (like abolishment of proton gradients) results in inhibition of the vacuolar amino acid antiporters and consequent reduction of the vacuolar amino acid pools. To test this hypothesis amino acids in vacuoles of *vma2Δ* mutants (in which the V-ATPase is inactive) have been measured by HPLC and compared to the total intracellular amino acid pools. Surprisingly, inactivation of the V-ATPase does not affect the vacuolar pools of basic amino acids like arginine and lysine, but on the other hand, vacuoles of *vma2Δ* mutants contain less neutral amino acids like glutamine and leucine which were shown to be particular important for proper TORC1 signalling (Fig. 2A). Therefore, the same strain has been tested for Sch9 phosphorylation and, as expected, shows reduction of TORC1 activity in exponential phase (Fig. 2B). In line with the hypothesis that TORC1 senses two different amino acid pools, the response of this strain to CHX is normal confirming that the signal generated is rather cytosolic than vacuolar. In conclusion, these data

suggest that TORC1 responds to both amino acids available in the cytosol and amino acids stored in the vacuole.

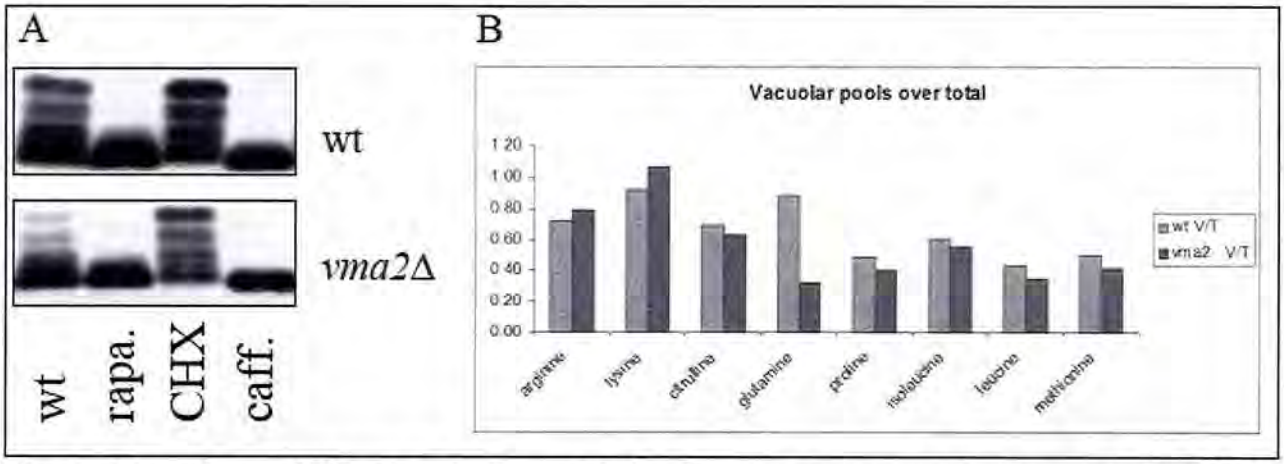

Fig. 2: Vacuolar Acidification is Essential for Proper Amino Acids Storage and TORC1 Signalling.
(A) Sch9 is partially dephosphorylated in exponential cells lacking a functional V-ATPase. Wild type and *vma2Δ* mutants were transformed with a plasmid expressing Sch9-HA from its endogenous promoter. Cells were grown exponentially in YPD containing 0.2% glutamine and treated with vehicle (exp), 200ng/ml rapamycin (rapa), 25 µg/ml cycloheximide (CHX) or 20 mM caffeine (caff) for 30 minutes. Cell extracts were subjected to SDS-PAGE, and immunoblots were probed with anti-HA antibodies.
(B) Amino acid pools are affected when the V-ATPase is inactivated. Wild type and *vma2Δ* mutants were grown exponentially in YPD. Total amino acids or amino acids isolated from intact vacuoles were determined by HPLC and represented by bars as ratios between vacuolar versus total pools. Data are representative of experiments repeated twice.

7.4. Autophagy recycles amino acids to maintain a basal TORC1 activity during starvation

Since also autophagy contributes to recycling amino acids via the vacuolar route during nitrogen starvation, it has been tested what happens to the TORC1 pathway under conditions of nitrogen deprivation. The first indication that the recycled amino acids are important to maintain a basal level of TORC1 activity comes from the observation that, nitrogen starvation causes a fast inactivation of TORC1 which is, however, transient. After one hour of nitrogen starvation a modest phosphorylation of Sch9 becomes slowly detectable, although the levels are lower when compared to exponentially growing cells (Fig. 3). This effect is dependent on the EGOC (it is not observable in a *gtr1Δ* mutant) and it is possibly due to amino acids that are recycled in the vacuole and eventually exported back into the cytoplasm (Yang et al., 2006). To further test the hypothesis that autophagy is important to maintain TORC1 activity during nitrogen starvation, the same experiment has been repeated under conditions in which autophagy is not induced after nitrogen starvation. While in *atg1Δ* mutants autophagy cannot be induced, in Pep4-lacking cells the autophagosomes

cannot be released and degraded in the vacuole. This effect can also be mimicked using PMSF that inhibits the vacuolar serine proteases thereby causing a block of degradation of autophagic bodies in the vacuole. The partial recovery of Sch9 phosphorylation following dephosphorylation triggered by nitrogen starvation is abolished or delayed when autophagy was blocked by either loss of Pep4 or by PMSF (Fig. 3B-C). These results indicate that autophagy, and thus vacuolar amino acids, are important regulators of TORC1 activity.

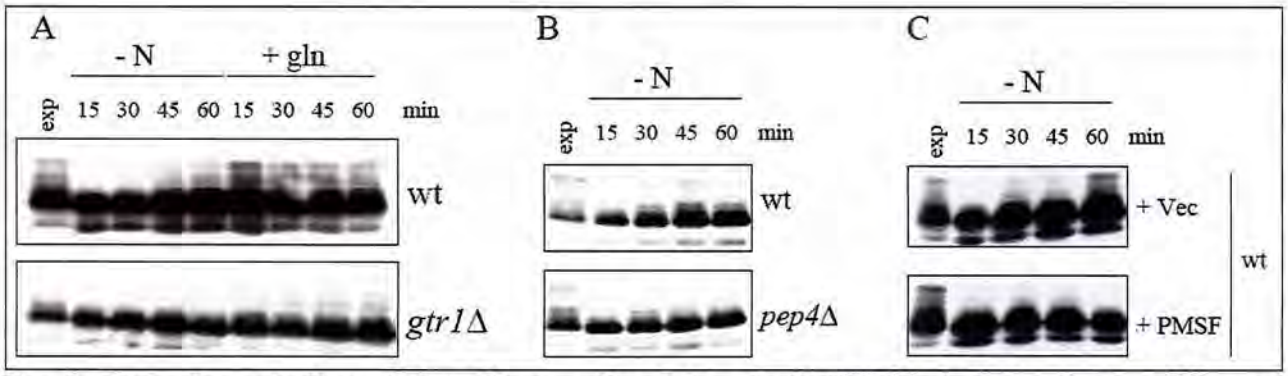

Fig. 3: Autophagy is Essential to Keep a Basal Level of TORC1 Activity During Nitrogen Starvation.

(A) Prototrophic wild type and *gtr1Δ* mutants were exponentially grown in complete medium, washed and starved for nitrogen. Glutamine was added after one hour of starvation to restart growth and sample were collected during starvation and recovery every 15 minutes for Sch9 analysis.

(B) Prototroph wild type and *pep4Δ* mutants were exponentially grown in complete medium, washed and starved for nitrogen for one hour. Samples were collected every 15 minutes for Sch9 analysis.

(C) Prototroph wild type was exponentially grown in complete medium and pre-treated with PMSF (1mM). After 30 minutes, cells were washed and resuspended in nitrogen-free medium with PMSF for one hour. Samples were collected every 15 minutes for Sch9 analysis. As control, a separate aliquot of the same wild-type was processed in the same manner but treated with vehicle instead of PMSF.

7.5. Conclusions

Together with the results obtained in Chapter I, the data presented in this chapter confirm the role of TORC1 as intracellular sensor of amino acids. When cells are grown in amino acid rich media, the SPS sensor is responsible for sensing the presence of extracellular amino acids and activates a signalling cascade that ultimately causes the sorting of the amino acid permeases Gnp1 and Agp1 to the plasma membrane which take up amino acids from the environment. It is not clear if TORC1 responds to extracellular amino acids. However, some data presented in Chapter I do not favour such a model: in prototrophic yeast cells growing in synthetic media lacking all amino acids, TORC1 is active. On the other hand, TORC1 is activated in such cells following CHX treatment, an

event that triggers a robust and fast increase of the intracellular amino acids indicating that those amino acids are key for TORC1 activity. Finally, a link between the SPS sensor and the TORC1 pathway has been already observed: inactivation of TORC1 by rapamycin triggers inactivation of two different steps in the SPS signalling cascade (Eckert-Boulet et al., 2006; Shin et al., 2009). A reverse mechanism by which the SPS sensor activates TORC1 in response to external amino acids remains to be studied.

When amino acids are taken up by actively growing cells they can have different fates. They can be directly incorporated into proteins, or they can be temporarily stored in the vacuole for successive utilization. In any case, yeast cells must always monitor the quantity of amino acids present in both pools to decide whether they should enter into quiescence or into a new cell cycle. In this chapter, TORC1 has been proposed to sensw the cytoplasmic amino acids pools. Given its localization at the vacuolar membrane it is tempting to speculate that TORC1 responds to the vacuolar amino acid pools. Some experiments presented in this chapter favour this model. Firstly, loss of three vacuolar exporters (Avt3, Avt4 and Avt6), which is expected to result in increased vacuolar amino acid pools (hypothesis to be verified), causes rapamycin resistance and secondly, loss of the V-ATPase subunit Vma2, that causes a reduction of different vacuolar amino acid pools, results in TORC1 inactivation.

It was previously shown that the EGOC acts as activator of TORC1 in response to amino acid. Since TORC1 may respond to both cytoplasmic and vacuolar amino acid pools, the new question to be answered is: what is the contribution of the EGOC in the intracellular amino acid sensing network? It is still premature to answer this question and it is only possible to speculate. Nevertheless, deletion of all three *AVT3, AVT4* and *AVT6* results in increased resistance to rapamycin but on the other hand it can partially bypass the inability of *ego3*Δ and *gtr1*Δ mutants to recover after rapamycin exposure. This result shows that the contribution of the EGOC in TORC1 activation in response to vacuolar amino acid pools is minimal. Additionally, Gtr1 is essential to maintain basal activity of TORC1 during nitrogen deprivation. As explained in section 5.7.5., starvation for nitrogen inhibits TORC1 and induces autophagy, a catabolic process that consists in degradation of proteins in the vacuole with subsequent recycling of the resulting amino acids into the cytoplasm. In wild-type cells, autophagy is essential for partial re-activation of TORC1 during nitrogen starvation. In the absence of Gtr1, TORC1 cannot be reactivated suggesting that the EGOC mediates mostly the cytoplasmic amino acid signal to TORC1.

Interpretation of the results presented in this chapter is merely speculative. Additionally, the model by which TORC1 is activated by both amino acid pools with the EGOC mediating the signal that originates from the cytoplasmic pool needs further verifications. The tools available at the moment might be quite limiting for those purposes but, for instance, it would be interesting to test the distribution of amino acids in the *avt3Δ avt4Δ avt6Δ* triple mutant or to test if in isolated vacuoles the EGOC is still active (*e.g.* Gtr1 binding to Ego1) and binds to TORC1.

8. Chapter III:

Approaches to identify new EGOC and/or TORC1 regulators

8.1. Introduction

In Chapter I it was shown that the GEF Vam6 controls the EGOC which, in turn, binds and activates TORC1 in response to amino acids (Binda et al., 2009). The integrity of the whole pathway is essential to prevent cells to display the so called *ego* phenotype that is defined as inhability to exit a rapamycin-induced growth arrest and rapamycin hypersensitivity (Dubouloz et al., 2005). It is possible that other regulatory proteins may exist that regulate the function of this pathway at different levels:

A. In addition to Vam6, other proteins may control the activation state of the EGOC. For instance, a GAP for Gtr1 would be an interesting protein to be discovered, as well as a GEF and a GAP for Gtr2. Notably, also the amino acid sensor that activates the EGOC in response to amino acids has not been clearly identified yet.
B. It is also possible that the EGOC is not the only activator of TORC1. There might be other proteins or other protein complexes that activate TORC1 in response to different cellular stimuli.
C. Since TORC1 activity prevents cells to display the *ego* phenotype, it might be interesting to understand which TORC1 targets are responsible for this phenotype.

Therefore, several genome-wide screens have been designed to identify new TORC1 regulators or effectors as listed above. In the screen described in section 8.2, an overexpression collection was used to identify proteins that, when overexpressed, render yeast cells unable to recover from a rapamycin treatment. The identified proteins (listed in Table 1) are potential negative regulators of the EGOC-TORC1 pathway and might act in the positions T.1 in Figure 1.

A second screen was already described in Chapter 1. Vam6 was identified in a *s*ynthetic *d*osage *l*ethality (SDL) screen (Measday et al., 2005). Deletion of *VAM6* exacerbates the slow growth phenotype of a strain overexpressing the GDP-locked form of Gtr1 from the *GAL1* inducible promoter. In section 8.3. the tables 2.a. (synthetic lethal interactors) and 2.b. (suppressors) list the complete set of results that were not described in Chapter 1 and that might play a role in the positions marked with 2.a. and 2.b. of the model in Fig. 1. In particular, suppressors of the slow growth phenotype of cells overexpressing Gtr1-GDP locked form are potential inhibitors of the EGOC-TORC1 pathway. On the other hand, the deletion mutants that showed a synthetic lethal interaction with the bait are likely to be activators of EGOC and/or TORC1.

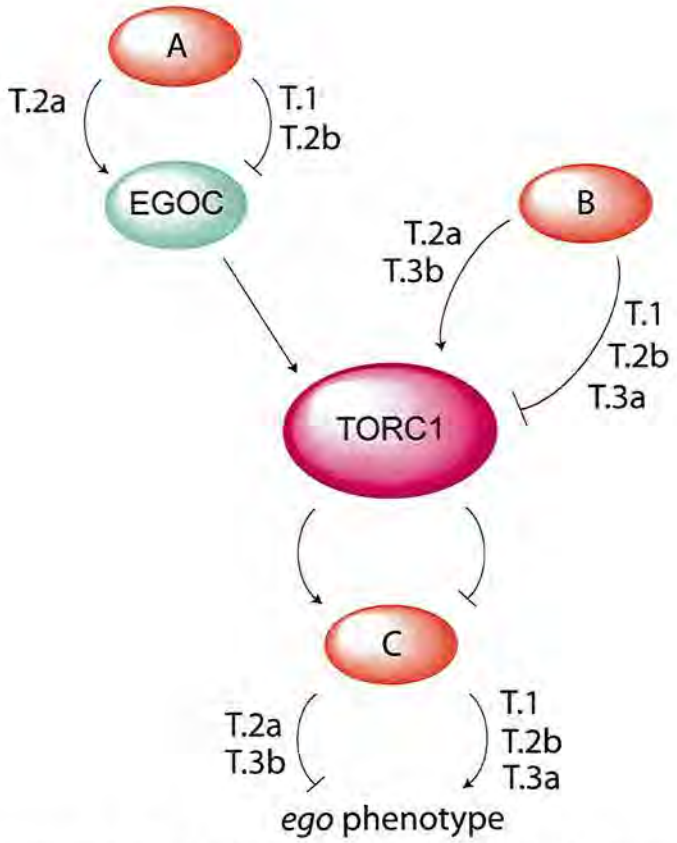

Fig. 1: Positions of Regulators that Might be Identified with the Different Genetic Screens. Different screening procedures have been developed in this study to identify possible regulators of the EGOC (A) and TORC1 (B) or downstream effectors of TORC1 (C). In this model to every activatory or inhibitory arrow is associated the name of the tables listed in this section containing the identified proteins that play a potential role in the corresponding positions in the pathway.

Finally, in section 8.4., it is presented a third screen aimed to find proteins that, when overexpressed, are *s*ynthetic *l*ethal (SL) with or can suppress the *ego* phenotype of *gtr2*Δ. SL interactors are listed in Table 3.a and are believed to act as inhibitors of the TORC1 pathway, while suppressors (Table 3.b) might be activators of TORC1 (corresponding positions are indicated in Fig. 1). It is unlikely to identify upstream regulators of the EGOC in this screen because this complex is inactivated by loss of Gtr2.

8.2. Screening for proteins that render cells unable to recover from rapamycin treatment when overexpressed

The EGOC was originally discovered in a screen for deletion mutants that are unable to regrow after rapamycin treatment (Dubouloz et al., 2005). In a similar approach, the *GAL1*-ORF-TAP collection containing around 5800 genes under the control of the *GAL1* inducible promoter (Gelperin et al., 2005), has been used here to screen for proteins that could confer an *ego* phenotype

when overexpressed. In this screen negative regulators of the EGOC-TORC1 pathway (like a GAP for Gtr1, or inhibitors of TORC1) were expected to be found. The collection was first replicated on Gal/Raff plates for one day to induce overexpression of the proteins and growth was subsequently arrested replicating the array, for an additional day, on the same medium containing 200ng/ml of rapamycin. After the release from the rapamycin block cells were allowed to grow for two days on rapamycin-free Gal/Raff plates and the clones that failed to resume growth were visually scored and re-tested independently. A total of 120 clones were identified with defects in recovery after rapamycin treatment. The mutants were isolated, plasmid were retrieved and retransformed individually into a wild-type. The resulting strains were then manually tested again for their *ego* phenotype. In control experiment, the same strains were grown on media without rapamycin and clones showing reduced growth rates were discarded as false positives (Fig. 2). Among the original 120 clones, 20 resulted to be true positives. These are listed in Table 1.

Interestingly, overexpression of Gln3 impairs cells for recovery after rapamycin treatment. This is in line with the observation that deletion of *GLN3* suppresses the phenotype of the *ego* mutants (Chapter 1, Supplemental Data I). Vps60 is a vacuolar protein involved in late endosome to vacuole transport (Kranz et al., 2001) and, by interacting with the multivesicular body (MVB) protein Vta1 (Shiflett et al., 2004), stimulates the MVB pathway (Azmi et al., 2008). It may be speculated that overexpression of Vps60 triggers an increased flux of protein degradation via the MVB pathway thereby probably degrading some key components of the EGOC-TORC1 pathway. Iml1 is another very interesting protein of unknown function that localizes at the vacuolar membrane. It is the closest yeast homolog of mammalian DEPTOR, a novel inhibitor of mTOR pathway involved in multiple myeloma cells (Peterson et al., 2009). It is not clear if Iml1 inhibits the EGOC or TORC1 in yeast, further analysis is absolutely necessary and it would be interesting to test this protein for its ability to act as Gtr1 GAP or Gtr2 GEF. The other results are not completely clear although the leucyl tRNA synthetase Cdc60 is quite interesting since it is possible to speculate that overexpression of this protein results in depletion of the cytosolic leucine, causing EGOC inactivation. Sky1 is a conserved Ser/Thr kinase that phosphorylates pre-mRNA splicing factors (Siebel et al., 1999). It was predicted to be phosphorylated in a TORC1-dependent manner leading to the hypothesis that, given the prevalence of introns in RP genes, TORC1 could play a direct role in ribosomal biogenesis (Huber et al., 2009). The other proteins listed in Table 1 are apparently unrelated to the function of the EGOC, even though it is possible that their overexpression causes side effects that inhibit somehow TORC1 activity.

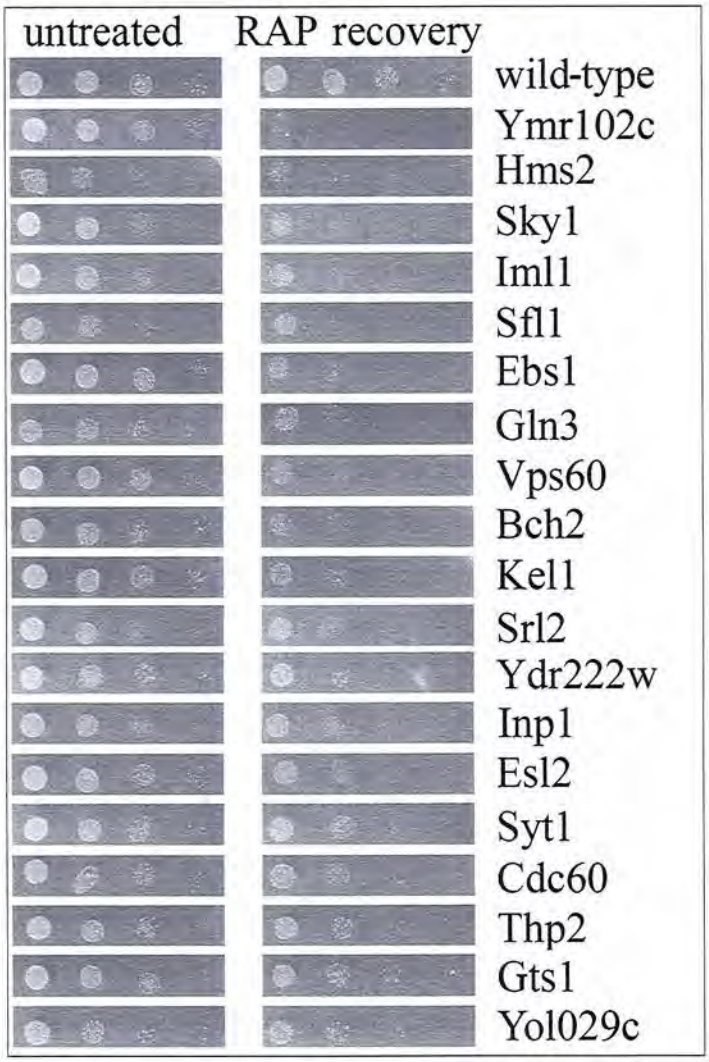

Fig.2: Confirmation of the *ego* Phenotype of Strains Overexpressing a Corresponding *GAL1*-ORF-TAP Gene.
Colonies that were impaired for recovery after a rapamycin block were from the *GAL1*-ORF-TAP collection. The plasmids contained in these strains were isolated and re-transformed into a BY4741 wild-type. The resulting strains were grown on Raff/Gal medium for proper protein induction and divided in two aliquots. The first aliquot was directly diluted and spotted on Raff/Gal containing plates (untreated control), the other half was treated with rapamycin for 6 hours, washed, diluted, spotted on the same medium and grown for two days to assay their ability to recover.

In the figure only the confirmed positives (also listed in Table 1) are shown together with a wild-type.

Table 1. Proteins that Cause an *ego* Phenotype when Overexpressed from the *GAL1* promoter

ORF Protein	Function
Ymr102c	Protein of unknown function; transcription is activated by paralogous transcription factors Yrm1p and Yrr1p along with genes involved in multidrug resistance; mutant shows increased resistance to azoles
Hms2	Protein with similarity to heat shock transcription factors; overexpression suppresses the pseudohyphal filamentation defect of a diploid mep1 mep2 homozygous null mutant
Sky1	SR protein kinase (SRPK) involved in regulating proteins involved in mRNA metabolism and cation homeostasis; similar to human SRPK1
Iml1	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the vacuolar membrane
Sfl1	Transcriptional repressor and activator; involved in repression of flocculation-related genes, and activation of stress responsive genes; negatively regulated by cAMP-dependent protein kinase A subunit Tpk2p
Ebs1	Protein involved in inhibition of translation and nonsense-mediated decay; interacts with cap binding protein Cdc33p and with Nam7p; localizes to P-bodies upon glucose starvation
Gln3	Transcriptional activator of genes regulated by nitrogen catabolite repression (NCR), localization and activity regulated by quality of nitrogen source
Vps60	Cytoplasmic and vacuolar membrane protein involved in late endosome to vacuole transport; required for normal filament maturation during pseudohyphal growth; may function in targeting cargo proteins for degradation; interacts with Vta1p
Bch2	Member of the ChAPs family of proteins (Chs5p-Arf1p-binding proteins: Bch1p, Bch2p, Bud7p, Chs6p), that forms the exomer complex with Chs5p to mediate export of specific cargo proteins, including Chs3p, from the Golgi to the plasma membrane
Kel1	Protein required for proper cell fusion and cell morphology; functions in a complex with Kel2p to negatively regulate mitotic exit, interacts with Tem1p and Lte1p; localizes to regions of polarized growth; potential Cdc28p substrate
Srl2	Protein of unknown function; overexpression suppresses the lethality caused by a rad53 null mutation
Ydr222w	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
Inp1	Peripheral membrane protein of peroxisomes involved in peroxisomal inheritance
Esl2	Protein of unknown function that may interact with ribosomes, based on co-purification experiments; green fluorescent protein (GFP)-fusion protein localizes to the nucleus and cytoplasm; predicted to contain a PINc domain
Syt1	Guanine nucleotide exchange factor (GEF) for Arf proteins; involved in vesicular transport; suppressor of ypt3 mutations; member of the Sec7-domain family
Cdc60	Cytosolic leucyl tRNA synthetase, ligates leucine to the appropriate tRNA
Thp2	Subunit of the THO complex, which connects transcription elongation and mitotic recombination, and of the TREX complex, which is recruited to activated genes and couples transcription to mRNA export; involved in telomere maintenance
Gts1	Protein that localizes to the nucleus and is involved in transcription regulation; also localizes to actin patches and plays a role in endocytosis; N-terminus contains an ARF-GAP domain and C-terminus contains a Gln-rich domain
Yol029c	Putative protein of unknown function; identified as interacting with Hsc82p and Hsp82p in high-throughput two-hybrid screens

8.3. Screening for mutants that can aggravate or suppress the slow growth phenotype of strain overexpressing the Gtr1^{GDP} allele

The Vam6 GEF displays GDP-release activity on Gtr1 and was thus suggested to be an activator of Gtr1. It was genetically identified in this study in an SDL screen since its deletion can exacerbate the slow growth phenotype of a strain overexpressing the GDP-locked form of Gtr1 from the *GAL1* inducible promoter. This genome-wide screen (described in Chapter I) also identified a number of deletion mutants (listed in Table 2.a) which were moderately sick when combined with the *GAL1*-Gtr1^{GDP} allele. In parallel to the screen for the SDL interactions, it was also looked for deletion mutants that could suppress the slow growth phenotype of the cells expressing the GDP-locked allele of Gtr1 (Table 2.b). The wild-type bait carrying the GDP-locked form of Gtr1 under the *GAL1* inducible promoter was crossed with the yeast deletion collection in order to create deletion strains carrying this construct. After the final pinning the collection was replicated on Raff/Gal media and growth rates were scored after two days. Large colonies were considered to be formed by cells harbouring a potential suppressor mutation, while small ones (or undetectable ones) were considered significant of synthetic lethal interactions.

Table 2.a. Deletion Strains that are Synthetic Lethal with a Wild Type Overexpressing the GDP-Locked Form of Gtr1

ORF Protein	Function
Vam6	Vacuolar protein that plays a critical role in the tethering steps of vacuolar membrane fusion by facilitating guanine nucleotide exchange on small guanosine triphosphatase Ypt7p
Gtr1	Cytoplasmic GTP binding protein and negative regulator of the Ran/Tc4 GTPase cycle; component of GSE complex, which is required for sorting of Gap1p; involved in phosphate transport and telomeric silencing; similar to human RagA and RagB
Vps16	Subunit of the vacuole fusion and protein sorting HOPS complex and the CORVET tethering complex; part of the Class C Vps complex essential for membrane docking and fusion at Golgi-to-endosome and endosome-to-vacuole protein transport stages
Ssd1	Protein with a role in maintenance of cellular integrity, interacts with components of the TOR pathway; ssd1 mutant of a clinical S. cerevisiae strain displays elevated virulence
Inp53	Polyphosphatidylinositol phosphatase, dephosphorylates multiple phosphatidylinositols; involved in trans Golgi network-to-early endosome pathway; hyperosmotic stress causes translocation to actin patches; contains Sac1 and 5-ptase domains
Vps17	Subunit of the membrane-associated retromer complex essential for endosome-to-Golgi retrograde protein transport; peripheral membrane protein that assembles onto the membrane with Vps5p to promote vesicle formation
Vps1	Dynamin-like GTPase required for vacuolar sorting; also involved in actin cytoskeleton organization, late Golgi-retention of some proteins, regulating peroxisome biogenesis
Bre5	Ubiquitin protease cofactor, forms deubiquitination complex with Ubp3p that coregulates anterograde and retrograde transport between the endoplasmic reticulum and Golgi compartments; null is sensitive to brefeldin A
Arf1	ADP-ribosylation factor, GTPase of the Ras superfamily involved in regulation of coated vesicle formation in intracellular trafficking within the Golgi; functionally interchangeable with Arf2p

Table 2.b. List of Deletion Strains that can Suppress the Slow Growth Phenotype of a Wild Type Overexpressing the GDP-Locked Form of Gtr1

ORF Protein	Function
Vps30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy and Complex II is required for vacuolar protein sorting; ortholog of the higher eukaryotic gene Beclin 1
Vps34	Phosphatidylinositol 3-kinase responsible for the synthesis of phosphatidylinositol 3-phosphate; forms membrane-associated signal transduction complex with Vps15p to regulate protein sorting; activated by the GTP-bound form of Gpa1p
Yck3	Palmitoylated, vacuolar membrane-localized casein kinase I isoform; negatively regulates vacuole fusion during hypertonic stress via phosphorylation of Vps41p; shares essential functions with Hrr25p; regulates vesicle fusion in AP-3 pathway
Ypk2	Protein kinase with similarity to serine/threonine protein kinase Ypk1p; functionally redundant with YPK1 at the genetic level; participates in a signaling pathway required for optimal cell wall integrity; homolog of mammalian kinase SGK
Aps2	Small subunit of the clathrin-associated adaptor complex AP-2, which is involved in protein sorting at the plasma membrane; related to the sigma subunit of the mammalian plasma membrane clathrin-associated protein (AP-2) complex
Sly41	Protein involved in ER-to-Golgi transport
Ssp120	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
Sro77	Protein with roles in exocytosis and cation homeostasis; functions in docking and fusion of post-Golgi vesicles with plasma membrane; homolog of Sro7p and Drosophila lethal giant larvae tumor suppressor; interacts with SNARE protein Sec9p
Aut2	Conserved cysteine protease required for autophagy; cleaves Atg8p to a form required for autophagosome and Cvt vesicle generation
Gea1	Guanine nucleotide exchange factor for ADP ribosylation factors (ARFs), involved in vesicular transport between the Golgi and ER, Golgi organization, and actin cytoskeleton organization; similar to but not functionally redundant with Gea2p
Apg9	Transmembrane protein involved in forming Cvt and autophagic vesicles; cycles between the phagophore assembly site (PAS) and other cytosolic punctate structures, not found in autophagosomes; may be involved in membrane delivery to the PAS
Aps3	Small subunit of the clathrin-associated adaptor complex AP-3, which is involved in vacuolar protein sorting; related to the sigma subunit of the mammalian clathrin AP-3 complex; suppressor of loss of casein kinase 1 function
Snx41	Sorting nexin, involved in the retrieval of late-Golgi SNAREs from the post-Golgi endosome to the trans-Golgi network; interacts with Snx4p
Gga1	Golgi-localized protein with homology to gamma-adaptin, interacts with and regulates Arf1p and Arf2p in a GTP-dependent manner in order to facilitate traffic through the late Golgi
Yck2	Palmitoylated plasma membrane-bound casein kinase I isoform; shares redundant functions with Yck1p in morphogenesis, proper septin assembly, endocytic trafficking; provides an essential function overlapping with that of Yck1p
Nyv1	v-SNARE component of the vacuolar SNARE complex involved in vesicle fusion; inhibits ATP-dependent Ca(2+) transport activity of Pmc1p in the vacuolar membrane
Sso1	Plasma membrane t-SNARE involved in fusion of secretory vesicles at the plasma membrane and in vesicle fusion during sporulation; forms a complex with Sec9p that binds v-SNARE Snc2p; syntaxin homolog; functionally redundant with Sso2p
Bst1	GPI inositol deacylase of the ER that negatively regulates COPII vesicle formation, prevents production of vesicles with defective subunits, required for proper discrimination between resident ER proteins and Golgi-bound cargo molecules
Arv1	Protein functioning in transport of glycosylphosphatidylinositol intermediates into the ER lumen; required for normal intracellular sterol distribution and for sphingolipid metabolism; similar to Nup120p and C. elegans R05H5.5 protein

It is interesting to note that deletion of *VPS16*, which plays a part together with Vam6 in the HOPS complex, can aggreviate the slow growth phenotype of cells overexpressing the dominant negative form of Gtr1. This probably means that Vps16 might also have a dual role in vacuolar fusion and EGOC activity maybe stabilizing or controlling Vam6. It is also interesting to notice that deletion of *YCK3* suppresses the slow growth phenotype of the bait tested. Yck3 is a protein kinase localized at the vacuolar membrane that phosphorylates the HOPS complex subunit Vps41/Vam2 thereby regulating vacuolar fusion (LaGrassa and Ungermann, 2005). It has been proposed that in the absence of Yck3, both Vps41 and Vam6 are more associated with vacuoles and thus they are believed to be more active on their targets. Ssd1 is a protein that cooperates with Tco89 for the maintenance of cellular integrity (Reinke et al., 2004). It displays a synthetic lethal interaction with Tor1, thus its synthetic lethality with $Gtr1^{GDP}$ is not surprising. This result may indicate that efficient EGOC signalling is essential in conditions of altered cell integrity. On the other hand, the fact that deletions of *VPS34* and *VPS30* suppress a phenotype associated with lower TORC1 activity is quite surprising. *VPS34* and *VPS30* code for two subunits of a phosphatidylinositol 3-kinase complex that produces phosphatidylinositol 3-phosphate (PIP3) and that is activated by the GTP-bound form of Gpa1 (Slessareva et al., 2006). In mammalian cells the phosphatidylinositol 3-kinase (PI3K) is an upstream activator of mTOR (Gingras et al., 1998), but in yeast it has never been shown if PIP3 is an upstream molecule essential for TORC1 signalling. However, Vps34 interacts genetically with Tor1 since *tor1*Δ strains become sick upon loss of Vps34 (Zurita-Martinez et al., 2007). This indicates either that Vps34 is an upstream regulator of TORC1 or that Vps34 and Tor1 act in parallel pathways on the same downstream targets. The fact that in this screen Vps30 and Vps34 came up as possible negative regulators in the TORC1 network (Fig. 1) is astonishing and needs further investigations. Yck2 is a protein involved in the control of SPS sensor pathway. Together with Yck1, phosphorylates Ptr3 in response to amino acid binding to the Ssy1 receptor (Liu et al., 2008), but it was shown also to be required for proper septin organization (Robinson et al., 1999). In this contest, it is unlikely the inactivation of the SPS sensor pathway (that is supposed to result in less amino acid uptake) suppresses a phenotype that results from inactivation of TORC1. On the other hand it is possible to speculate that proper septin organization is important in condition of low TORC1 activity.

8.4. Screening for proteins that, when overexpressed, are SL with deletion of *GTR2* or can suppress the *ego* phenotype of *gtr2*Δ mutants

The same *gtr2*Δ bait used in the previous study (Dubouloz et al, 2005) was additionally crossed with the arrayed *GAL1*-GST-ORF collection created by the lab of B. Andrews (Sopko et al., 2006). The purpose of this screen was to identify proteins that act on TORC1 in parallel to the EGOC. If an overexpressed protein can suppress the *ego* phenotype it is likely to act positively in the TORC1 pathway and hopefully is an activator of TORC1. On the other hand, a protein that upon overexpression renders *gtr2*Δ strains inviable represents a potential inhibitor of TORC1 pathway. The final pinning of the screen resulted in *gtr2*Δ strains overexpressing a given protein from the *GAL1* promoter. All strains were grown for two days on Raff/Gal plates and slow growing strains (cleaned from the false positives, see below) are listed in Table 3.a. At this stage, the collection was replicated on rapamycin containing media for one day and then released. The strains that were able to restart growing after two days were independently tested and the positives are listed in Table 3.b. The original collection was grown on Raff/Gal media to score for false positive strains displaying a slow growth phenotype on their own.

Table 3.a. List of Proteins that Cause Lethality When Overexpressed in a *gtr2*Δ Mutant

ORF Protein	Function
Smp1	Putative transcription factor involved in regulating the response to osmotic stress; member of the MADS-box family of transcription factors
Mcm2	Protein involved in DNA replication; component of the Mcm2-7 hexameric complex that binds chromatin as a part of the pre-replicative complex
Cmd1	Calmodulin; Ca++ binding protein that regulates Ca++ independent processes (mitosis, bud growth, actin organization, endocytosis, etc.) and Ca++ dependent processes (stress-activated pathways), targets include Nuf1p, Myo2p and calcineurin
Yar061w	Pseudogene with similarity to Flo1p, which is a lectin-like protein involved in flocculation
Bug1	Cis-golgi localized protein involved in ER to Golgi transport; forms a complex with the mammalian GRASP65 homolog, Grh1p; mutants are compromised for the fusion of ER-derived vesicles with Golgi membranes
Okp1	Outer kinetochore protein, required for accurate mitotic chromosome segregation; component of the kinetochore sub-complex COMA (Ctf19p, Okp1p, Mcm21p, Ame1p) that functions as a platform for kinetochore assembly
Ost6	Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins; similar to and partially functionally redundant with Ost3p

Table 3.b. List of Proteins that Can Suppress the *ego* Phenotype of *gtr2*Δ Mutant When Overexpressed

ORF Protein	Function
Ure2	Nitrogen catabolite repression transcriptional regulator that acts by inhibition of GLN3 transcription in good nitrogen source
Gzf3	GATA zinc finger protein and Dal80p homolog that negatively regulates nitrogen catabolic gene expression by competing with Gat1p for GATA site binding; function requires a repressive carbon source; dimerizes with Dal80p and binds to Tor1p
Gdh3	NADP(+)-dependent glutamate dehydrogenase, synthesizes glutamate from ammonia and alpha-ketoglutarate; rate of alpha-ketoglutarate utilization differs from Gdh1p; expression regulated by nitrogen and carbon sources
Ape3	Vacuolar aminopeptidase Y, processed to mature form by Prb1p
Dal81	Positive regulator of genes in multiple nitrogen degradation pathways; contains DNA binding domain but does not appear to bind the dodecanucleotide sequence present in the promoter region of many genes involved in allantoin catabolism
YJR096W	Putative xylose and arabinose reductase; member of the aldo-keto reductase (AKR) family; GFP-fusion protein is induced in response to the DNA-damaging agent MMS
Tsc13	Enoyl reductase that catalyzes the last step in each cycle of very long chain fatty acid elongation, localizes to the ER, highly enriched in a structure marking nuclear-vacuolar junctions, coimmunoprecipitates with elongases Fen1p and Sur4p
Pph22	Catalytic subunit of protein phosphatase 2A, functionally redundant with Pph21p; methylated at C terminus; forms alternate complexes with several regulatory subunits; involved in signal transduction and regulation of mitosis
Tes1	Peroxisomal acyl-CoA thioesterase likely to be involved in fatty acid oxidation rather than fatty acid synthesis; conserved protein also found in human peroxisomes; TES1 mRNA levels increase during growth on fatty acids
Prs4	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase, synthesizes PRPP, which is required for nucleotide, histidine, and tryptophan biosynthesis; one of five related enzymes, which are active as heteromultimeric complexes
Pyk1	Pyruvate kinase, functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration
Rrd2	Activator of the phosphotyrosyl phosphatase activity of PP2A,peptidyl-prolyl cis/trans-isomerase; regulates G1 phase progression, the osmoresponse, microtubule dynamics; subunit of the Tap42p-Pph21p-Rrd2p complex

Overexpression of Gdh3 can probably result in higher levels of glutamate and/or glutamine reinforcing the hypothesis that TORC1 is controlled by amino acids and/or by good nitrogen sources. Most of the other suppressors of *gtr2*Δ are already known to be part of the TORC1 network. Ure2 inhibits Gln3 during growth on good nitrogen sources and, like Gzf3 (Soussi-Boudekou et al., 1997), negatively controls expression of the NCR-sensitive genes (Kulkarni et al., 2001). It is possible to speculate that if Ure2 is overexpressed during rapamycin treatment, Gln3 cannot enter into the nucleus and thus cannot start induction of the NCR-sensitive genes. In a similar manner, overexpression of Gzf3, that competes with Gat1 for promoter binding, might prevent the other GATA transcription factor Gat1 to bind to promoters of the NCR-sensitive genes. Those speculations lead to the hypothesis that altered transcription of one or more NCR-sensitive genes might cause the *ego* phenotype in the *egoc* mutants. This is in line with results shown in Supplemental Data I, Fig. 1, where deletion of *GLN3* suppresses the *ego* phenotype of *gtr1*Δ and

gtr2Δ mutants. Dal81 is involved in different nitrogen degradation pathways (Bricmont et al., 1991). It is possible that overexpression of this gene causes an increase in nitrogen production with a subsequent boost of TORC1 activity. Ape3 is an interesting protein because it is a vacuolar aminopeptidase (Yasuhara et al., 1994) and thus its overexpression might increase vacuolar protein degradation followed by recycling of amino acids that impinge on TORC1. On the other hand, Tsc13 is reported to be a protein essential for piecemeal microautophagy of the nucleus (PMN) (Kvam et al., 2005). Probably, overexpression of Tsc13 and induction of PMN might have a similar effect as overexpression of Ape3.

8.5. Conclusions

In this section different screens aimed to identify new regulators of the EGOC-TORC1 pathway have been described and the most interesting results are summarized in a speculative model shown in Fig. 3.

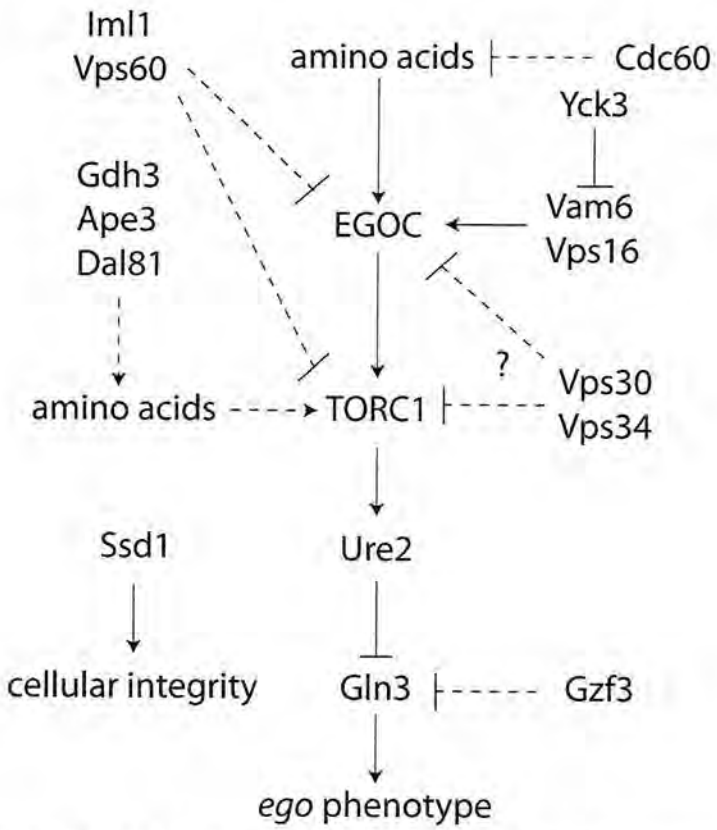

Fig. 3: Summary of the EGOC-TORC1 Network Including the Screening Results.
See the text for the details, dotted arrows represent putative interactions.

Among the newly identified potential regulators of the EGOC-TORC1 there are several interesting clones that need further investigations in order to add substantial knowledge to the field.

(i) New potential upstream regulators of the EGOC or of TORC1, like Iml1 and Vps60, have been identified. It is necessary now to proceed with additional studies and understand the exact role that they play in the TORC1 network. In particular, Iml1 is the closest yeast homolog to mammalian DEPTOR, an inhibitor of mTORC1. An interesting issue is whether it might work as a GAP for Gtr1.

(ii) Additionally, three genes (*APE3*, *DAL81* and *GDH3*) that code for proteins involved in amino acid homeostasis show synthetic interactions with TORC1 bypassing the requirement of the EGOC in this pathway. This further indicates that TORC1 might sense amino acids via other pathways parallel to the EGOC. It would be interesting to test if overexpression of these three genes in a *gtr2*Δ mutant might also bypass the inability of TORC1 to properly phosphorylate Sch9.

(iii) The role of Vam6 as activator of EGOC is further reinforced by the fact that the HOPS complex subunit Vps16 came out in the same screen as Vam6 even though it was a weaker positive and that loss of Yck3, inhibitor of the HOPS complex, results in an opposite effect.

(iv) The inability of recover from a rapamycin block of the *ego* mutants is apparently caused by a hyperactivation of the NCR pathway. Overexpression of inhibitors of this pathway (Ure2 and Gzf3) suppresses the *ego* phenotype of *gtr2*Δ cells, while overexpression of Gln3 causes an *ego* phenotype. It would be therefore interesting in further studies to identify the targets of the NCR pathways that are responsible for the *ego* phenotype.

Additionally to these genes, other putative interactors have been identified in this study. However, given their roles in cellular functions completely unrelated to TORC1 it was difficult to locate them in the model in fig. 3.

9. Chapter IV: General discussion and perspectives

9.1. New regulators of TORC1 in response to nutrients

The EGOC, composed of Ego1, Ego3, Gtr1 and Gtr2, has already been identified independently in two different studies. Dubuoloz and colleagues (2005) proposed that the EGOC may act in parallel or possibly upstream of TORC1 in regulation of microautophagy, while the group of Kaiser showed that the EGOC is necessary to sort the general amino acid permease Gap1 to the plasma membrane (Gao and Kaiser, 2006). While the molecular function of the non-conserved proteins Ego1 an Ego3 is not clear, Gtr1 and Gtr2 are two small G-proteins with homology to mammalian Rag GTPases. In this work, the EGOC subunits were shown, to some extent like their mammalian counterpart, to be upstream regulators of TORC1. In particular it has been shown that the activation state of the EGOC in yeast is mainly dictated by the nucleotide bound form of Gtr1, the subunit that binds to TORC1 (probably via Kog1) in a leucine dependent manner. EGOC is proposed to be a direct activator of TORC1 in yeast. These data fill in the gap between amino acids and TORC1 signalling to some extent. Binding of the EGOC to TORC1 is dependent on the nucleotide loading status of Gtr1, which is also controlled by the GEF Vam6, and which is crucial for TORC1 activation. On the other hand, the role of Gtr2 is still unclear. Despite it is essential for proper EGOC function, it seems to act inhibitory towards TORC1. Different roles can be attributed to Gtr2: it might act in an independent unidentified inhibitory pathway, or it might destabilize the GTP-bound form of Gtr1 acting indirectly as a GAP but not as *G*DP *d*issociation *i*nhibitor (GDI) since in the presence of Gtr2, Vam6 can still displace the GDP from Gtr1 (Binda et al., 2009). The novel EGOC function presented here does not fit with its proposed involvement in Gap1 sorting as reported by the Kaiser group: Gap1 production and sorting to the plasma membrane are processes that occour when TORC1 activity is reduced (Chen and Kaiser, 2003). Thus, low TORC1 activity, like for example in the *egoc* mutants, should, in principle, favour newly synthesized Gap1 to be sorted to the plasma membrane. Moreover, in the BY4741 background (Binda et al., 2009), Gap1 is internalized from the plasma membrane for degradation when cells are shifted from a good to a poor nitrogen source, while in the background used by the group of Kaiser, Gap1 is sorted to the plasma membrane even during grown in ammonium containing media (Courchesne and Magasanik, 1983). Indirect involvement of Gap1 in the TORC1 signalling pathway was further excluded in this work and experiments showed that in the BY4741 background the EGOC does not control Gap1 sorting to the plasma membrane.

The exact nature of the stimulus that is sensed by the EGOC for proper TORC1 signalling remains to be uncovered. Amino acids, leucine in particular, have been shown to play an important role in

TORC1 activity both in yeast and higher eukaryotes, but it has not been tested whether the EGOC responds to other amino acids rather than leucine. Additionally, it is still not clear if EGOC and TORC1 respond preferentially to vacuolar or cytoplasmic pools of these amino acids. However, both pools need to be continuously monitored for proper coordination of protein synthesis with cell cycle progression. In case that growth conditions request high levels of protein synthesis, amino acids must be exported from the vacuole into the cytoplasm for quick utilization, otherwise they are stored in the vacuole. Notably, in a recent genome wide phosphoproteomic analysis, Avt1 was reported to be phosphorylated in a TORC1-dependent manner (Huber *et al.* 2009).

Some additional experiments have been performed to address the issue whether TORC1 responds preferentially to cytoplasmic or vacuolar amino acid pools. Inactivation of the V-ATPase renders cells unable to acidify the vacuole and to store properly some amino acids in the vacuolar lumen probably because of the abolishment of the electrochemical gradient across the vacuolar membrane. In V-ATPase-deficient strains, TORC1 activity is reduced during exponential phase, but those cells still respond properly to CHX treatment suggesting that TORC1 somehow responds to both vacuolar and cytoplasmic amino acid pools. Since Ego3 is predicted to contain a transmembrane domain, the EGOC is a good candidate for a vacuolar amino acid sensor. It has been previously shown that lack of three vacuolar amino acid exporters Avt3, Avt4 and Avt6, results in vacuolar accumulation of tyrosine and other neutral amino acids (Yang et al., 2006). Deletion of the genes coding for those proteins increased the cells' resistance to rapamycin and partially suppressed the *ego* phenotype of *ego3Δ* and *gtr1Δ* strains. This argues against a model in which the EGOC is the TORC1 sensor for vacuolar amino acids. On the other hand, EGOC is essential to favour minimal TORC1 activity during nutrient starvation conditions that induce autophagy. Nitrogen starvation triggers a rapid TORC1 inactivation that results in increased autophagic flux with consequent recycling of amino acids into the cytoplasm via Avt3, Avt4 and Atg22 (Yang *et al.* 2006) and partial reactivation of TORC1. This reactivation process depends on the presence of a functional EGOC and is abolished, indeed, in conditions in which the release of autophagosomes in the vacuole is inhibited. The data presented in this work favour a model by which TORC1 responds to both intracellular nutrients and amino acids. Given its localization at the vacuolar surface, TORC1 is hence proposed to sense both vacuolar and cytoplasmic amino acids, with the EGOC mediating rather the cytoplasmic amino acid signal (Fig.1).

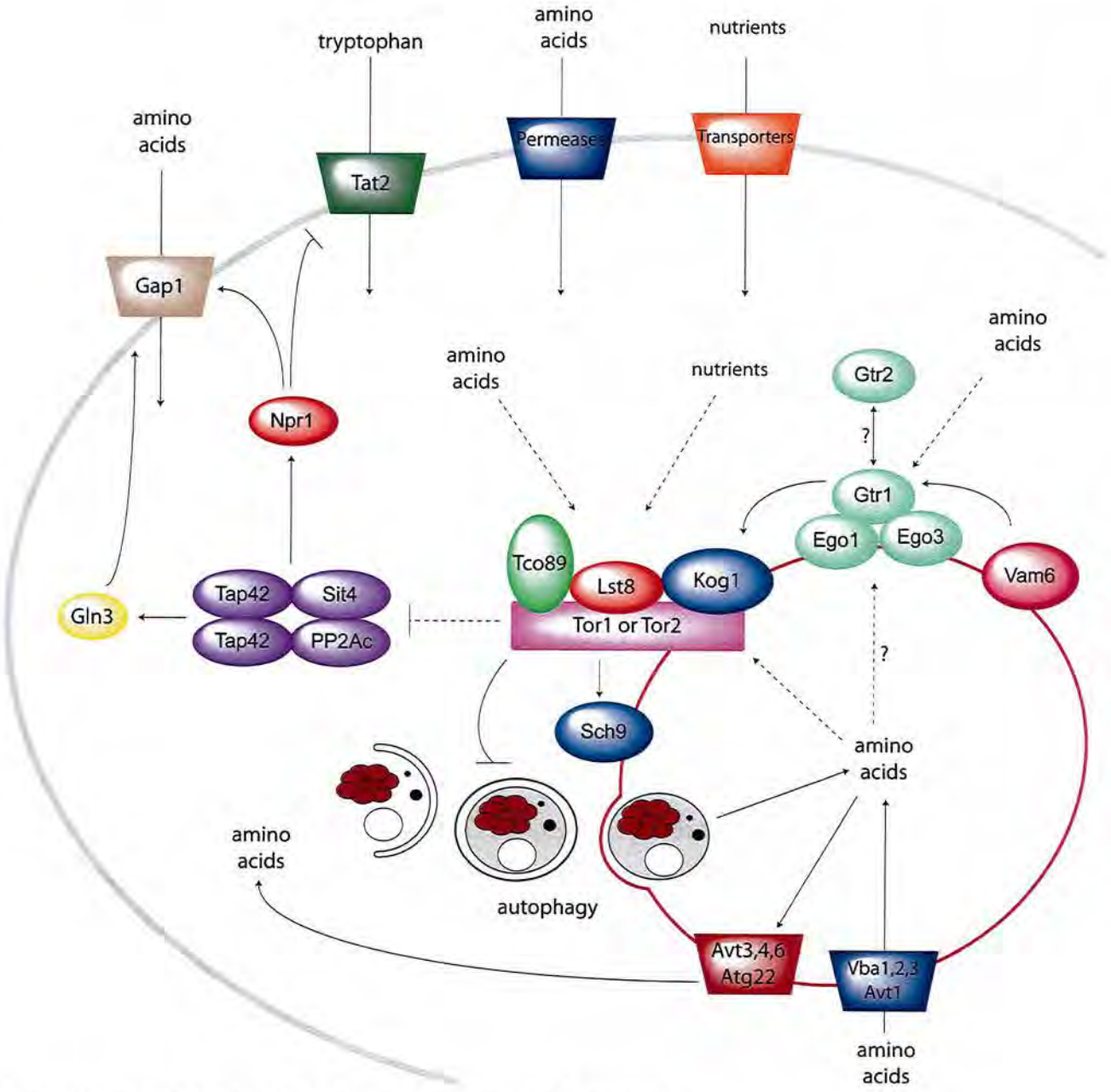

Fig.1: Working Model for Amino Acid Control of TORC1.
Amino acids have central roles in the cell. They are taken up by specific or general transporters and used immediately for protein synthesis or stored in the vacuole. Moreover, they trigger activation of the vacuolar localized EGOC dictating the GTP-loading status of Gtr1 which is also controlled by Vam6. In turn, the EGOC activates TORC1 which controls growth, protein synthesis, activity of several amino acid permeases, autophagy and other cellular processes.

As shown in different studies, TORC1 controls localization and activity of several permeases mainly via its effector Npr1. This kinase is inhibited by TORC1 and gets fully activated after rapamycin treatment or nitrogen starvation favouring, via unknown mechanisms, sorting of Gap1 to the plasma membrane and degradation of the tryptophan permease Tat2. In *egoc* and *tco89Δ* mutants the steady-state levels of Npr1 are dramatically reduced but it seems that the low detectable amount of Npr1 in these cells is in the active form because of less TORC1 activity. Surprisingly,

egoc mutants still display no missorting defect of Tat2 and/or Gap1 during growth on rich nitrogen sources indicating that the EGOC has no role in this process.

In rich conditions, cells are healthy because TORC1 favours growth stimulating both uptake of amino acids and protein synthesis. In case of a shift to less favourable growth conditions, TORC1 is quickly downregulated resulting in both internalization of specific permeases and sorting of Gap1 to the plasma membrane. Growth slows down and autophagy flow is increased with consequent recycling of amino acids from the vacuole into the cytoplasm. Most of the proteins needed for growth are probably degraded and the recycled amino acids are used for proper induction of the G_0 program. At the same time, the cytoplasmic amino acids that are recycled via autophagy might trigger, via EGOC, a basal level of TORC1 activity necessary to maintain minimal protein synthesis rates. This might explain why autophagy and *ego* mutants are starvation sensitive.

9.2. The Vam6 GEF acts upstream in the TORC1 pathway

Vam6 is a previously characterized GEF that controls vacuolar fusion and inheritance acting on the small Rab GTPase Ypt7 (Wurmser et al., 2000). In this study Vam6 is also shown to control Gtr1 activity since it displays *in vitro* GDP release activity on Gtr1. The first genetic interaction between Gtr1 and Vam6 was discovered in a genome wide screen aimed to find viable deletion mutants that are able to exacerbate the slow growth phenotype of wild type cells overexpressing a GDP-locked allele of Gtr1. Further genetic and biochemical assays clearly showed that the activation state of Gtr1 is controlled by Vam6 and, as a consequence, in cells lacking Vam6 the EGOC is unable to activate TORC1 in response to amino acids. In this study Vam6 can be located with quite some confidence upstream of EGOC and TORC1 because: i) *vam6Δ* mutants phenocopy *gtr1Δ* and *gtr2Δ* strains (*e.g.* with respect to their inability to recover after rapamycin-induced growth arrest and their impairment in Sch9 phosphorylation under all conditions tested); ii) overexpression of Vam6 confers rapamycin resistance in a wild type, but not in the absence of Gtr1 or Tco89, and suppresses the slow growth phenotype of cells overexpressing the GDP-bound allele of Gtr1; iii) expression of Tor1 hyperactive alleles suppresses the rapamycin sensitivity of *vam6Δ* mutants; iv) Gtr1 interacts with Vam6 in co-immunoprecipitation experiments; v) Vam6 shows GEF activity over Gtr1; vi) in the absence of Vam6 the *in vivo* interaction between Ego1 and Gtr1 (indicator of the active state of Gtr1) is very weak. This is in line with previous discoveries showing that Vam6 controls TORC1 activity via an unknown mechanism (Yang et al., 2006).

Vam6 is part of the vacuolar localized HOPS complex that controls the dynamics of vacuolar fusion and inheritance via the GTPase Ypt7 (Rab7 in mammals). Cells with altered HOPS complex function are easily identifiable by the huge number of small vacuoles that they contain. It is rather unlikely that the effects on TORC1 activity observed upon loss of *VAM6* are due to the abnormal vacuolar morphology of this strain since *ypt7Δ* cells display normal TORC1 activity and proper assembly of the EGOC. Vam6 is proposed to have two distinct intracellular roles: it regulates vacuolar morphology via the Rab GTPase Ypt7, and controls TORC1 activity dictating the nucleotide-bound state of Gtr1. It is not known whether those two functions are mutually exclusive. All the players in the linear pathway found in this study (*e.g.* Vam6-EGOC-TORC1-Sch9) co-localize at the vacuolar surface and occasionally also to punctate structures adjacent to the vacuole that are supposed to be late endosomes (Cabrera *et al.* 2009; Gao and Kaiser 2006). Moreover, given the role of Vam6 in ensuring vacuolar fusion in different growth phases, it probably coordinates vacuolar fusion via Ypt7, with TORC1 signalling via Gtr1. Conversely, it is also reasonable that there are two completely separate (but always vacuolar localized) pools of Vam6, one responding to amino acids for Gtr1-TORC1 activation, and the other one responds to an unknown stimulus that triggers vacuolar fusion at contact sites between two adjacent vacuoles (Fig.2).

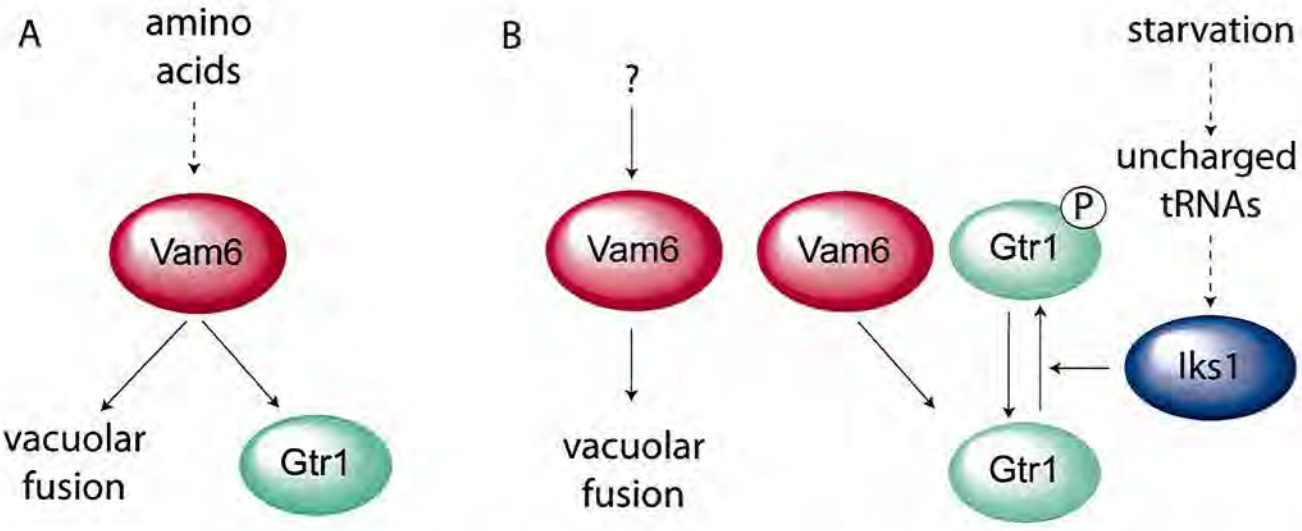

Fig.2: Speculative Models for the Dual Function of Vam6 in EGOC Activation and Control of Vacuolar Fusion Events.
(A) Vam6 might respond directly to amino acids and coordinate vacuolar fusion with EGOC activation.
(B) Vam6 both controls vacuolar fusion and Gtr1 in an amino acid independent manner. Starvation indirectly causes an increase of the uncharged tRNAs. This effect could be sensed by the Gcn4-like kinase Iks1 that phosphorylates Gtr1 rendering it less available for Vam6. When amino acids are present, Iks1 is maybe inactive and Gtr1 can be thus targeted by Vam6.

How can Vam6 integrate the amino acid signal for Gtr1 activation? A few hypotheses can be made to answer this question. It is possible that Vam6 does not respond directly to amino acids and that this signal is sensed by another upstream protein that modulates the availability of Gtr1 for Vam6. In this respect it is important to mention that Gtr1 is predicted to be phosphorylated by Iks1 (Ptacek et al., 2005), a protein kinase of unknown function that shares strong homology with the kinase Gcn2 that is regulated by the levels of uncharged tRNAs (Dong et al. 2000). Thus, it is possible that Iks1 senses, like Gcn2, uncharged tRNAs, which are increased following amino acid limitation, and phosphorylates Gtr1 thereby reducing its affinity for Vam6. Another hypothesis is that Vam6 senses directly amino acid availability and regulates both vacuolar fusion, in order to organize the storage compartment, and TORC1 activity to promote cell cycle progression.

9.3. Similarities and differences between yeast and mammalian TORC1 signalling in response to amino acids

The localization data of the yeast EGOC are for some aspects consistent with the results obtained in mammalian cells: the Rag GTPases nicely colocalize with the lysosomal marker Rab7. Rab7 is the mammalian homologue of Ypt7 and it is controlled by mVam6 for lysosome clustering and fusion processes. It would therefore be interesting to see whether hVam6 shows GEF activity on mammalian RagA/B. However there is a stricking difference between yeast and mammalian cells concerning the working model of the Rag GTPases. In mammals, activity of the Rag proteins is believed to affect localization of mTORC1 at the lysosomal Rheb-positive membranes in order to be fully activated by the known activator Rheb. On the other hand, in yeast, localization of the proteins present in the pathway does not change upon leucine deprivation, moreover Rhb1, the yeast homologue of mammalian Rheb, does not seem to be an activator of TORC1. It is possible that the role of the Rag GTPases has diverged during evolution and that the yeast EGOC is important for TORC1 activity rather than for its localization. It would be possible to check this hypothesis testing the *in vitro* ability of yeast purified TORC1 to phosphorylate Sch9 in the presence of EGOC purified from different yeast strains expressing the nucleotide-locked alleles of Gtr1. Nevertheless, it is not possible to exclude that the EGOC controls also the localization of TORC1 in response to particular conditions that have not been tested yet. Given the differences between yeast and mammalian cells, this still remains an interesting topic which is worth studying in the future.

10. Materials and methods

List of Strains Used in Chapter 1

Strain	Genotype	Source	Figure
BY4741	*MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*	Euroscarf	
BY4742	*MATα*; *his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0*	Euroscarf	3C
YL515	[BY4741/2] *MATα*; *his3Δ1, leu2Δ0, ura3Δ0*	This study	1A, B, D; 2; 3B; 4A, B; 5A, E, G-K ; 6B-D; S1; S2
YL516	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0*	This study	3C, D; 5B-D, F; 6A
MB25	[YL515] *MATα*; *ego1Δ::HIS3*	This study	1A
MB26	[YL515] *MATα*; *ego3Δ::HIS3*	This study	1A
MB27	[YL515] *MATα*; *gtr1Δ::HIS3*	This study	1A, D; 2A
MB28	[YL515] *MATα*; *gtr2Δ::HIS3*	This study	1A; 2A
MB34	[YL515] *MATα*; *tco89Δ::HIS3*	This study	1A, D
MB32	[YL516] *MAT***a**; *gtr1Δ::kanMX4*	This study	1C; 3D; 5A-D; S1, 3
MB33	[YL516] *MAT***a**; *gtr2Δ::kanMX4*	This study	2B; 5B, C; S1
YJU531	[YL516] *MAT***a**; *tco89Δ::natMX*	This study	5D
MP07-1D	[YL516] *MAT***a**; *vam6Δ::kanMX4*	This study	5A-C, F, K; 6A, C, D
MP02-7B	[YL515] *MATα*; *gtr1Δ::kanMX4, tco89Δ::HIS3*	This study	1B, C
MP06-8B	[YL515] *MATα*; *gtr1Δ::kanMX4, gtr2Δ::kanMX4*	This study	3A
MB35	[YL515] *MATα*; *gln3Δ::HYGR*	This study	S1
MB36-2C	[YL516] *MAT***a**; *gtr1Δ::kanMX4, gln3Δ::HYGR*	This study	S1
MP01-4D	[YL516] *MAT***a**; *gtr2Δ::kanMX4, gln3Δ::HYGR*	This study	S1
YJU537	[YL516] *MAT***a**; *EGO1-GFP::kanMX6*	This study	6A, B
MP56	[YL516] *MAT***a**; *TCO89-GFP::kanMX6*	This study	6A, B
KT1960	*MAT***a**; *his3, leu2, ura3, trp1*	Pedruzzi et al. 2003	
FD34	[KT1960] *MAT***a**; *his3, leu2, ura3, trp1, KOG1-TAP::HIS3*	This study	4B, E
FD35	[KT1960] *MAT***a**; *his3, leu2, ura3, trp1, TCO89- TAP::HIS3*	This study	4A, D
MP52-2A	[YL516] *MAT***a**; *TOR1-D330-3xGFP*	This study	6A, B
Y07050	[BY4741] *MAT***a**; *gap1Δ::kanMX4*	Euroscarf	2C, D
Y00575	[BY4741] *MAT***a**; *ypt7Δ::kanMX4*	Euroscarf	5A, K; 6C, D
MB56	[BY4741] *MAT***a**; *GTR1-GFP::HIS3*	Huh et al. 2003	6A
NMY51	*MAT***a**; *his3Δ200, trp1-901, leu2-3,112, ade2,* LYS2::(lexAop)4-HIS3, ura3::(lexAop)8- lacZ, ade2::(lexAop)8-ADE2, GAL4	Dual systems Biotech AG	4C

List of Plasmids Used in Chapter I

Plasmid	Description	Source	Figure
pRS413	CEN, *HIS3*	Brachmann et al. 1998	1; 2A, B; 3B, C; 5B-E; 6A, C; S1
pRS415	CEN, *LEU2*	Brachmann et al. 1998	1; 2; 3A-C; 5B-D; 6B-D; S1-3
pRS416	CEN, *URA3*	Brachmann et al. 1998	1D; 2B-D; 3A, B; 5B; 6; S2
YCplac33	CEN, *URA3*	Gietz et al. 1988	1C; 3D
pJU660	[pRS415] *GTR1*	This study	1B; 3A
pJU656	[pRS415] $GTR1^{S20L}$	This study	1B; 3A
pMB1483	[pRS415] $GTR1^{Q65L}$	This study	1B; 3A
pJU651	[pRS416] *GTR2*	This study	3A
pJU654	[pRS416] $GTR2^{S23L}$	This study	3A
pJU655	[pRS416] $GTR2^{Q66L}$	This study	3A
pMB1393	[YCplac33] *Teton-GTR1*	This study	1C; 3D; S3
pMB1394	[YCplac33] $Teton\text{-}GTR1^{Q65L}$	This study	1C; 3D; S3
pMB1395	[YCplac33] $Teton\text{-}GTR1^{S20L}$	This study	1C; 3D
pMB1344	[YCplac33] *GTR1-TAP*	This study	5I, K
pMB1371	[YCplac33] $GTR1^{S20L}$ *-TAP*	This study	5I
pMB1372	[YCplac33] $GTR1^{Q65L}$ *-TAP*	This study	5I
YCpIF2	CEN, *LEU2, GAL1-GST*	Foreman et al. 1994	
pMB1348	[YCpIF2] *GAL1-GST-GTR1*	This study	4A, B,
pMB1579	[YCpIF2] $GAL1\text{-}GST\text{-}GTR1^{Q65L}$	This study	4A, B
pMB1580	[YCpIF2] $GAL1\text{-}GST\text{-}GTR1^{S20L}$	This study	4A, B
pMB1667	[YCpIF2] *GAL1-GST-GTR1, leu2::URA3*	This study	4D, E
pMB1668	[YCpIF2] $GAL1\text{-}GST\text{-}GTR1^{Q65L}$, *leu2::URA3*	This study	4D, E
pVW1146	[YIpIF2] *GAL1-GTR1*	This study	5A, E
pVW1148	[YIpIF2] $GAL1\text{-}GTR1^{S20L}$	This study	5A, E
pJU1064	[pRS413] $SCH9^{T750A}\text{-}HA_5$	Urban et al. 2007	2C, D; 3D; 6D; S2; S3
pJU1030	[pRS416] $SCH9^{T750A}\text{-}HA_5$	Urban et al. 2007	1A,B; 3C; 5C; S1
pCDV1084	[YCpIF2] *ADH1-GST-EGO1*	Dubouloz et al. 2005	5I, K
pMPG1574	2μ, $Teton\text{-}HIS_6\text{-}HA_3$, *URA3*	This study	5D, E, H
pMPG1576	[pMPG1574] $Teton\text{-}HIS_6\text{-}HA_3\text{-}VAM6$	This study	5D, E, G, H
pPL132	[pRS315] $HA_3\text{-}TOR1$	Reinke et al. 2006	1D; 5F
pPL155	[pRS315] $HA_3\text{-}TOR1^{A1957V}$	Reinke et al. 2006	1D; 5F
pPL156	[pRS315] $HA_3\text{-}TOR1^{I1954V}$	Reinke et al. 2006	1D; 5F
pPL157	[pRS315] $HA_3\text{-}TOR1^{W2176R}$	Reinke et al. 2006	1D
pMB1650	[pGEX3X] *RAS2*	This study	5G, H
pMB1651	[pGEX3X] *YPT7*	This study	5G, H
pJU1032	[pGEX6P] $GTR1\text{-}HIS_6$	This study	5G, H
pJU1015	[pET28a] *GTR2*	This study	5G
pJU793	[pRS416] *GFP-SCH9*	Urban et al. 2007	6A
pJOD10	CEN, *URA3, GAL1-GAP1-GFP*	Nikko et al. 2003	2A
pMB1730	[pRS413] *ADH1-RFP-VAM6*	This study	6B
pMB1379	[YCplac33] *MET15*	This study	2C, D; 6C, D
pMPG1728	[pRS413] *CYC1-GFP-VAM6*	This study	6A
pDL2-Alg5	2μ, *ADH1-HA-NUBG, TRP1*	Dualsystems	4C

pCabWT	CEN, *CYC1-CUB-LEXA, LEU2*	Dualsystems	4C
pNP1711	[pCabWT] *CYC1-TCO89-CUB-LEXA*	This study	4C
pNP1712	[pCabWT] *CYC1-EGO1-CUB-LEXA*	This study	4C
pPR3-N	2μ, *CYC1-NUBG-HA, TRP1*	Dualsystems	
pNP1689	[pPR3-N] *CYC1-NUBG-HA-GTR1*	This study	4C
pNP1690	[pPR3-N] *CYC1-NUBG-HA-GTR1^{Q65L}*	This study	4C
pNP1691	[pPR3-N] *CYC1-NUBG-HA-GTR1^{S20L}*	This study	4C

List of Strains Used in Supplemental Data II (SD II) and Chapter II (CH. II)

Strain	Genotype	Source	Figure
BY4741	*MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*	Euroscarf	SD II 3,5; CH. II 1,2,3
BY4742	*MATα; his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0*	Euroscarf	
Y05078	[BY4741] *MAT***a**; *ego1Δ::kanMX4*	Euroscarf	SD II 3,5; CH. II 1
Y03214	[BY4741] *MAT***a**; *ego3Δ::kanMX4*	Euroscarf	SD II 3; CH. II 1
Y06522	[BY4741] *MAT***a**; *gtr1Δ::kanMX4*	Euroscarf	SD II 3,5; CH. II 1,3
Y04793	[BY4741] *MAT***a**; *gtr2Δ::kanMX4*	Euroscarf	SD II 3
Y02072	[BY4741] *MAT***a**; *tco89Δ::kanMX4*	Euroscarf	SD II 3,5
Y11812	[BY4742] *MATα; pep12Δ::kanMX4*	Euroscarf	SD II 5
Y17050	[BY4742] *MATα; gap1Δ::kanMX4*	Euroscarf	SD II 5
MB11-4B	[BY4741/2] *MAT***a**; *pep12Δ::kanMX4, ego1Δ::kanMX4*	This study	SD II 5
MB14-1A	[BY4741/2] *MATα; pep12Δ::kanMX4, gtr1Δ::kanMX4*	This study	SD II 5
MB16-1A	[BY4741/2] *MATα; pep12Δ::kanMX4, tco89Δ::kanMX4*	This study	SD II 5
MB19-1A	[BY4741/2] *MATα; gap1Δ::kanMX4, ego1Δ::kanMX4*	This study	SD II 5
YL515	[BY4741/2] *MATα; his3Δ1, leu2Δ0, ura3Δ0*	This study	SD II 1,2,6
MB25	[YL515] *MATα; ego1Δ::HIS3*	This study	SD II 1,2
MB26	[YL515] *MATα; ego3Δ::HIS3*	This study	SD II 1,2
MB27	[YL515] *MATα; gtr1Δ::HIS3*	This study	SD II 1,2
MB28	[YL515] *MATα; gtr2Δ::HIS3*	This study	SD II 1,2
MB34	[YL515] *MATα; tco89Δ::HIS3*	This study	SD II 1,2
KT1960	*MAT***a**; *his3, leu2, ura3, trp1*	Pedrizzi et al. 2003	SD II 3,4
KT1961	*MATα; his3, leu2, ura3, trp1*	Pedrizzi et al. 2003	
FD34	[KT1960] *MAT***a**; *KOG1-TAP-HIS3*	This study	SD II 6
MB47-1A	[KT1960] *MAT***a**; *tco89Δ::kanMX4, KOG1-TAP-HIS3*	This study	SD II 6
MB48-1A	[KT1960] *MAT***a**; *tor1Δ::kanMX4, KOG1-TAP-HIS3*	This study	SD II 6
FD30	[KT1961] *MATα; tor1Δ::kanMX4*	This study	SD II 3,4
FD27	[KT1961] *MATα; tco89Δ::kanMX4*	This study	SD II 3,4
FD33	[KT1960] *MAT***a**; *gtr1Δ::natMX*	This study	SD II 3,4
CDV212	[KT1961] *MATα; gtr2Δ::natMX*	Dubouloz *et al.* 2005	SD II 3,4
Y02098	[BY4741] *MAT***a**; *pep4Δ::kanMX4*	Euroscarf	CH. II 3
Y03266	[BY4741] *MAT***a**; *vma2Δ::kanMX4*	Euroscarf	CH. II 2
CDV313-12B	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, avt6Δ::kanMX4*	This study	CH. II 1
CDV313-12C	[BY4741/2] *MATα; his3Δ1, leu2Δ0, ura3Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4, avt6Δ::kanMX4*	This study	CH. II 1
CDV312-3D	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4*	This study	CH. II 1
CDV325-1A	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4, avt6Δ::kanMX4, ego3Δ::kanMX4*	This study	CH. II 1

CDV325-1C	[BY4741/2] *MATα; his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4, avt6Δ::kanMX4, ego3Δ::kanMX4*	This study	CH. II 1
CDV332-18A	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4, avt6Δ::kanMX4, gtr1Δ::kanMX4*	This study	CH. II 1
CDV332-7B	BY4741/2] *MATα; his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, avt3Δ::kanMX4, avt4Δ::kanMX4, avt6Δ::kanMX4, gtr1Δ::kanMX4*	This study	CH. II 1

List of Plasmid Used in Supplemental Data II (SD II) and Chapter II (CH. II)

Plasmid	Description	Source	Figure
pMB1348	[YCpIF2] *GAL1-GST-GTR1*	This study	SD II 6
pRL450	CEN, *LEU2, TRP1, HIS3*	This study	SD II 4
pJOD10	CEN, *URA3, GAL1-GAP1-GFP*	Nikko et al. 2003	SD II 4
pMB1379	[YCplac33] *MET15*	This study	SD II 4; CH. II 3
pAS103	2μ, *URA3, NPR1-3HA*		SD II 2
pAH099	CEN, *URA3, MAF1-3HA*	Huber et al. 2009	SD II 1
pAS55	2μ, *URA3*, 3HA-TAT2	Beck et al. 1999	SD II 3
pRS415	CEN, *LEU2*	Brachmann et al. 1998	CH. II 3
pJU1064	[pRS413] *SCH9* ^{T570A} *-5HA*	Urban et al. 2007	CH. II 2,3

Growth Conditions

Unless stated otherwise, prototroph *S. cerevisiae* strains were pre-grown overnight in synthetic medium without amino acids (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulphate, and 2% glucose). Before each experiment, cells were diluted to an OD_{600} of 0.2 in YPD medium supplemented with 0.2% of glutamine and grown until they reached an OD_{600} of 0.8. For amino acid deprivation experiments, strains that were specifically auxotrophic for one amino acid were grown to an OD_{600} of 0.8 on complete synthetic medium (SC; *e.g.* SD plus all amino acids), washed twice, and resuspended in starvation medium (SC-Leu, SC-Lys, or SC-His, lacking specifically one relevant amino acid). For nitrogen starvation, prototroph cells were grown to an OD_{600} of 1.0 on SD (without amino acids), washed twice, and resuspended in the same medium without ammonium sulphate. Low quality nitrogen media were SD media, which contained 10 mM urea or proline instead of ammonium sulphate.

Strains transformed with plasmids were pre-grown overnight in appropriate selective medium (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose, essential amino acids). Before each experiment, cells were diluted to an OD_{600} of 0.2 in YPD-medium supplemented with 0.2% of glutamine and grown until they reached an OD_{600} of 0.8.

Autotroph strains were pre-grown overnight in YPD medium. Before each experiment, cells were diluted to an OD_{600} of 0.2 in YPD medium supplemented with 0.2% of glutamine and grown until they reached an OD_{600} of 0.8.

2% galactose 1% raffinose media were used to induce expression of proteins under the GAL inducible promoter, 5 µg/ml of doxycycline was added to induce expression from the Tet promoter.

Low nitrogen media are composed of 2% glucose, 1.7 g/l of YNB(-N) (filtered from a 10X stock) plus 10 mM of urea or proline.

E. coli DH5α and BL21 strains were grown in LB supplemented with appropriate antibiotics for plasmid selection.

Sch9 Phosphorylation Analyses

To analyze $Sch9^{T570A}$-HA_5 C-terminal phosphorylation, we used the chemical fragmentation analysis as described previously (Urban et al. 2007). For quantifications of Sch9 phosphorylation, NTCB-cleaved extracts were separated by 7.5% SDS-PAGE followed by immunoblotting with anti-HA antibody 12CA5. The anti-HA antibody was detected with far-red fluorescent Alexa Fluor 680 dye-labeled secondary anti-mouse antibody (Invitrogen, A21057), and fluorescence intensity was measured using the Odyssey Infrared Imaging System (LI-COR).

Protein precipitation with TCA

10ml of exponentially growing cells were harvested by centrifugation, resuspended in 300µl of LB (180mM NaOH, 76µl/ml 2-mercaptoethanol) and incubated on ice for 10 minutes. 300µl of 50% TCA were then added and cells were incubated again on ice of 10 minutes. Cells were sedimented, washed twice with acetone, resuspended in 100µl of sample buffer and boiled for 5 minutes.

G-protein expression and purification from E. coli

Ypt7-GST, Ras2-GST, Gtr1-GST and GST alone were expressed in *E. coli* from pGEX-6P vectors. After a four hour induction with 1 mM IPTG a clarified bacterial lysate was prepared in PBS based buffer with 10% glycerol and 0.1% tween-20 and the fusion protein was bound to glutathione-Sepharose 4B resin (GE Healthcare) following standard procedures. Elution, if necessary, was performed with 500 µl of elution buffer (1x PBS, 20% glycerol, 10 mM reduced glutathione).

Co-immunoprecipitation experiments

For coprecipitation experiments using GST-tagged proteins, 250ml of cells were grown in appropriate media until an OD_{600} of 0.8. Cells were harvested by filtration, washed with water,

resuspended in 600µl of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, PPi) with 200µl of glass beads and lysed in a Fast-Prep apparatus. Extracts were clearified by two sequential steps of centrifugation (15 min, 13000 rpm) and GST-tagged proteins were purified using glutathione sepharose beads incubating the extracts with 50µl of washed resin for 2 hours. The resin was washed four times with the buffer, and GST-tagged proteins were eluted by heat denaturation in 50µl of Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125M Tris HCl) for 3 minutes.

Tco89-TAP and Kog1-TAP were purified from 250ml of cells grown in appropriate media until an OD_{600} of 0.8. Cells were harvested by filtration, washed with water, resuspended in 600µl of lysis buffer (PBS, 10% glycerol, 0.1% Tween-20, PPi) and broken as described previously. The clearified lysates were incubated for 90 minutes with magnetic beads (Invitrogen, 143-01) that were pre-coated over night in PBS with 1mg/ml IgG (Sigma, I5006) and in the presence of 1 M (NH4)2SO4 to facilitate the binding. The beads were washed four times with the buffer and TAP-tagged proteins were eluted by heat denaturation in 50µl of Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125M Tris HCl) for 3 minutes.

Extracts containing 6HIS-3HA-Vam6 were obtained by 250ml of cells grown in appropriate media until an OD_{600} of 0.8. Cells were harvested by filtration, washed with water, resuspended in 600µl of lysis buffer (PBS, 10% glycerol, 0.1% Tween-20, PPi), broken and clearified as previously described. The extracts were loaded in the presence of 10 mM EDTA on glutathione-Sepharose 4B resin (GE Healthcare) pre-decorated with GST-G-Proteins or GST alone. Gels were equilibrated according to the GST moiety.

Precipitated proteins subjected to standard immunoblot analysis for detection of coprecipitated proteins.

Vam6 purification for GEF release assay

For each series (4 samples), 6HIS-3HA-Vam6 (or 6HIS-3HA alone as control) was purified from 250ml of cells at an OD_{600} of 0.8. Cells were broken in lysis buffer (40mM Hepes pH 7.5, 5% glycerol, 0.1% NP-40, 150mM NaCl, PPi) and clarified lysates were incubated for 90 minutes with Ni-Agarose resin on rotation and eluted in 30µl of lysis buffer with 250mM of imidazole for 30 minutes. In these conditions 6µl of purified HIS_6-HA_3-Vam6 are needed for the reaction.

GDP release assay

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

Extraction of the vacuolar amino-acid pools (adapted from Oshumi *et al.* 1987)

Cells (~ 10 OD_{600}) were harvested by filtration and washed three times with distilled water. They were then resuspended and incubated for 10 minutes at room temperature directly onto the membrane in 3 ml of extraction buffer (2.5mM Potassium phosphate ; 0.6M sorbitol ; 10mM glucose ; 0.2mM $CuCl_2$; pH = 6.0). After permeabilization, cells were washed 3 times with 15ml of wash buffer (2,5mM Potassium phosphate; 200mM KCl ; 10mM $CaCl_2$; 5mM $MgCl_2$, pH = 6,0), resuspended in 1ml of water and boiled for 15 minutes. The suspension was centrifuged for 5 minutes at 13,000 rpm and the supernatant was filtered through 0.22μm filters.

Whole-cell amino acid analysis

Cells (~ 10 OD_{600}) were harvested by filtration and washed three times with distilled water. They were suspended in distilled water, boiled for 15 minutes and finally centrifuged 10 minutes at 13,000 rpm. The supernatant was finally filtered through 0.22μm filters.

Chromatographic separation and quantification of amino-acids

Vacuolar and whole-cell amino-acid pools were quantified with pulsed electrochemical detection after separation by anion exchange chromatography with a AAA-direct, Dionex Amino Acid

Analyser, using a sodium acetate gradient to increase the ionic strength. Three analyses were performed on independent samples.

Differential centrifugation and equilibrium density centrifugation (Kaiser *et al.*, 2002)
For gradients preparation the solutions with different sucrose concentration were prepared as follows (for 18 gradients):

	60%	50%	40%	30%	20%
70%sucrose	38.6ml	32.1ml	25.7ml	19.3ml	12.9ml
500mM Tris, pH8.5	4.5ml	4.5ml	4.5ml	4.5ml	4.5ml
1M KCl	1.125ml	1.125ml	1.125ml	1.125ml	1.125ml
1M $MgCl_2$	0.45ml	0.45ml	0.45ml	0.45ml	0.45ml
H_2O	0.325ml	6.825ml	13.225ml	19.625ml	26.025ml

2.23ml of each solution (starting with the most dense) was layered in a plastic Sorvall tube alternating every step with one hour of freezing at -80°C. Gradients were thawed over-night at room temperature before use.

Differential centrifugation was performed as described in Kaiser *et al.* 2002. 100ml of exponentially growing culture was harvested, washed once in 10mM sodium azide, 10mM potassium fluoride, 50mM tris pH 7.5. Cells were additionally washed in STE10 (10% sucrose, 10mM Tris pH 7.5, 10mM EDTA), resuspended in 500µl STE10 and lysed with glass bead. Cell debris was sedimented with a step of three minutes of centrifugation at 2000rpm and 300µl of extracts were carefully layered on top of the gradients. Samples were then centrifuged 17 hours at 4°C at 100000g in a SW50 rotor. Sixteen 330µl fractions were collected from the top of the gradient and frozen. For immunoblots, 50µl of each fraction were mixed with 50µl of sample buffer and incubated one hour at 37°C for solubilization. 50µl were loaded onto a gel for SDS-PAGE.

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Mpg1: multi-task enzyme involved in several processes. Associates also with the DNA and stimulates the proof-reading activity of the DNA Pol. In *mpg1*Δ mutants, cells are more prone to make any kind of mistake.
Tlr32: protein involved in ethanol metabolization. It interacts strongly with other proteins involved in the same function.
Ggr3: protein essential for proper function of Mpg1 and Mtt2. However, its precise role in the cell is still unknown.
Xnl9: anti stress response protein. When cells are stressed, Xnl9 still works hard completing its tasks, thus stimulating the other CDV proteins to behave the same way.
Svr4: binding partner of Mpg1 in the early phases of the cell cycle. In the Mpg1-Svr4 complex each protein helps the other to be stimulated by caffeine.

Dtr2: unusual protein that colocalizes with some components of the CDV pathway but apparently it does not share any function with them. Its role still remains unknown.

Flr5: recently discovered protein. It tightly binds to Mpg1 helping it to achieve its functions.

Mlk1: protein under direct control of Tlr32 even though it is not involved in ethanol metabolization. This aspect of Mlk1-Tlr32 interaction indicates that Tlr32 has also some other, yet to be discovered, functions.

Pnc2: protein with unknown function. Expression levels may change randomly during growth.

Pat3: chaperone, when the CDV pathway is inactive Pat3 targets all the components to ATP-free environments for relaxation.

Cdv1: main regulator of the CDV pathway. Oncogene: loss of Cdv1 causes massive miss-coordination of all the other proteins and cell cycle arrest in a random phase. Hyperactivation of Cdv1 triggers a considerable charge on the other proteins, uncontrolled growth for few replications that ends with a general cell collapse.

13. Declaration for the Faculty

Matteo Binda
Born in Lecco, Italy

Fribourg,

To whom it may concern

Subject: Thesis presented at the University of Fribourg, Switzerland, for obtaining the degree of Doctor rerum naturalium.

Madam, Sir,

I hereby declare that my thesis work entitled "The EGO Complex controls TORC1 in response to amino acids" was done my me and all data and concepts presented in this thesis result from no other sources other than my own work, unless stated otherwise.

Matteo Binda

13. Déclaration pour la Faculté

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Sujet: Thèse présentée à l'Université de Fribourg, Suisse, pour l'obtention du grade de Doctor rerum naturalium

Mesdames, Messieurs,

Par la présente, je certifie que j'ai rédigé ma these "The EGO Complex controls TORC1 in response to amino acids" moi-même et sur la base d'un travail personnel sans aide illicite.

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14. Curriculum Vitae

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Professional experiences:

University of Fribourg, Switzerland (Nov. 2005 – now)
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Katholieke Universiteit Leuven, Belgium (Sept. 2004 – Sept. 2005)
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Hospital A. Manzoni of Lecco, Italy (Sept. 2003)
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Education:

Ph.D. in Biology mention Biochemistry (Nov. 2005 – Dec. 2009)
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Master in Pharmacogenomical Biotechnology (Sept. 2003 – Nov. 2005)
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Thesis at the "Laboratorium voor Functionele Biologie", Katholieke Universiteit Leuven, Belgium from September 2004 until September 2005
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An EGOcentric view of TORC1 signaling

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Teaching of "Travaux Pratiques" to French- and English-speaking bachelor students
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Workshop in "Functional Genomics and Proteomics in Molecular Medicine" (Split, 3rd- 9th June 2007)
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Skills:

Molecular Biology: plasmid construction, cloning, sequencing, site-directed mutagenesis, marker swapping
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Genetics: yeast genetics: transformation, dissection, screen of DNA libraries, deletion and tagging of genes
High-Throughput Screens: two-hybrid, synthethic lethal, dosage rescue, chemogemomics screens

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