UPSTREAM REGULATION OF YEAST TOR COMPLEXES

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Dedicated to my family

To friends

To all the infinite possibilities that led me here and now

SUMMARY

Nitrogen is an essential component of living organism. Protein synthesis and biosynthesis of nitrogen containing molecules essential for growth, such as amino acids and NAD, strictly depend on the availability of the nitrogen source. Limiting amount of nitrogen limit cell growth. Exogenous amino acids and other nitrogenous compounds such as ammonium constitute the nitrogen source. The budding yeast *S. cerevisiae* can sense and utilize a total of 21 different nitrogenous compounds to sustain growth. The uptake and utilization of different nitrogen sources is hierarchical and subject to strict and complex regulation at the transcriptional, translation and post-translational level. In general, preferred nitrogen sources inhibit the uptake and catabolism of non-preferred nitrogen sources.

The target of rapamycin (TOR) is a conserved Ser/Thr protein kinase among eukaryotes controlling growth in response to nutrients and growth factors. TOR is found in two essential conserved multiprotein complexes named TOR complex 1 (TORC1) and TORC2. In yeast, TORC1 signaling is sensitive to nutrients, particularly to availability of the nitrogen source. Thus, TORC1 by promoting anabolic processes, such as protein synthesis and ribosome biogenesis, couples growth to the availability of the nitrogen source.

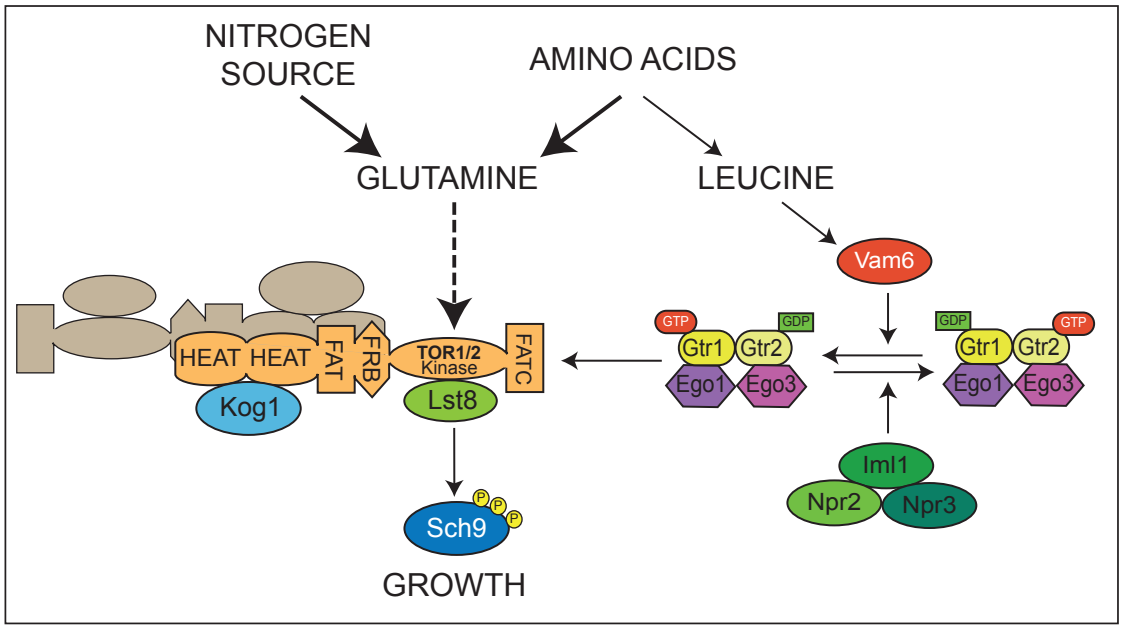
The sensing mechanism activating TORC1 in response to nitrogen source sufficiency is only poorly understood. Recent studies identified the EGO complex as intermediary component of the amino acid sensing pathway. In the presence of amino acids, particularly leucine, the EGO complex is activated and promotes TORC1 activity. The small GTP binding proteins Gtr1 and Gtr2 are part of the EGO complex. Gtr1 and Gtr2 form a heterodimeric complex. In response to amino acids Gtr1 is loaded with GTP and Gtr2 with GDP, leading to activation of the EGO complex. Several factors determine EGO complex activation by dictating the Gtr1/2 guanosine loading. Vam6 was proposed to act as guanosine exchange factor (GEF) towards Gtr1. The Npr2/Npr3/Iml1 complex was reported to act as GTPase activating protein (GAP) for Gtr1. Therefore, amino acid stimulation of TORC1 is decreased in the absence of Vam6 and increased in the absence of Iml1. Several observations point out that EGO complex signaling, alone, is not sufficient to explain TORC1 activation by the nitrogen source. For instance, 1) components of the EGO complex signaling are not essential; 2) ammonium starvation down-regulates growth even in cells where EGO complex signaling is hyperactivated. Therefore, nitrogen source and amino acids sufficiency might signal to TORC1 via distinct mechanisms.

In this study we analyze the effect of different nitrogen sources on TORC1 activity. We use the phosphorylation state of the direct TORC1 target

Sch9 as readout for TORC1 activity. We describe that preferred nitrogen sources activate TORC1 signaling stronger and better than non-preferred nitrogen source. TORC1 activation by preferred nitrogen sources is paralleled by an increase in glutamine synthesis and accumulation. Growth is increased in the presence of preferred nitrogen sources in a glutamine synthesis dependent way. Therefore, glutamine constitutes a metabolic input linking TORC1 activation in response to the quality of the nitrogen source to growth capacity. We find that EGO complex signaling is dispensable when a preferred nitrogen source is provided. TORC1 activation and growth increase are still induced in cells compromised for EGO complex signaling. Taken together, we demonstrate that nitrogen source and amino acid sufficiency act via discrete mechanisms to activate TORC1.

NOVEL FINDINGS

- TORC1 activity is rapidly induced by the quality of the nitrogen source
- TORC1-dependent phosphorylation sites on Sch9 are differently phosphorylated in vivo according to the quality of the nitrogen source
- Preferred nitrogen sources better sustain TORC1 activity over time than non-preferred nitrogen sources
- The ability of preferred nitrogen sources to induce and sustain TORC1 activity depends on their capacity to induce glutamine synthesis and accumulation
- Glutamine synthesis and accumulation constitute a metabolic input bridging the quality of the nitrogen source to TORC1 activation and growth capacity
- The metabolic input activates TORC1 independently of the EGO complex
- Amino acids and the nitrogen source activate TORC1 via independent mechanisms



Graphical abstract

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CONTENTS

1 INTRODUCTION

1.1 TOR discovery and early steps 1

1.1.1 Rapamycin and TOR 1

1.1.2 From TOR domains to TOR complexes 2

1.1.3 TORCs composition and localization 4

1.1.4 Perspective in TOR signaling research 7

1.2 TORC2 signaling 8

1.2.1 Downstream targets of TORC2 8

1.2.2 TORC2 upstream signaling 13

1.3 TORC1 signaling 14

1.3.1 Downstream targets of TORC1 14

1.3.2 TOR and nitrogen metabolism 21

1.3.3 TORC1 upstream signaling 29

1.4 Aim of the study 35

2 RESULTS

2.1 Capsule 39

2.2 Abstract 39

2.3 Introduction 40

2.4 Results 42

2.5 Discussion 56

2.6 Material and Methods 58

2.7 Tables 60

2.8 Additional Results 61

3 APPENDIX

3.1 Activation of mTORC2 by association with the ribosome 67

BIBLIOGRAPHY 81

LIST OF FIGURES

Figure 1: TORC2 signaling
Figure 2: TORC1 signaling
Figure 3: Nitrogen metabolism core reactions
Figure 4: mTORC1 upstream regulation
Figure 5: TORC1 upstream regulation
Figure 6: TORC1 rapidly phosphorylates Sch9 in response to the nitrogen source
Figure 7: The quality of the nitrogen source determines TORC1 activity
Figure 8: The quality of the nitrogen source couples TORC1 activation to cell growth
Figure 9: Intracellular glutamine constitutes a metabolic input to activate TORC1
Figure 10: MSX treatment blocks the metabolic input to TORC1
Figure 11: The metabolic input acts independently of the EGO complex in TORC1 activation
Figure 12: S711 and T737 are major TORC1-dependent phosphorylation sites of Sch9

ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
AGC	Protein kinase A, G, and C families
Akt/PKB	Rac protein kinase alpha / protein kinase B
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
Avo	Adheres voraciously to TOR
Bit61	Binding partner of TOR2
CHX	Cycloheximide
DEPTOR	DEP-domain-containing mTOR-interacting protein
DNA-PK	DNA-dependent protein kinase
EF3	Elongation factor 3
EGO	GTPase-containing complex for Gap1p sorting in the endosome
EGOC	EGO complex
ER	Endoplasmic reticulum
FAT	FRAP, ATM and TRRAP domain
FATC	FAT C-terminal, domain
FIT	Found in TOR, domain
FKBP12	FK506-binding protein 12
FPR1	FK506-sensitive proline rotamase
FRB	FKBP12-rapamycin binding domain
Gap1	General amino acid permease 1
GAP	GTPase-activating protein
GATOR	GTPase-activating protein activity toward RAGs
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
Gtr	GTP-binding protein resemblance
HEAT	Huntingtin, EF3, PP2A and the yeast kinase TOR1
HOPS	Homotypic vacuole fusion and vacuole protein sorting
HM	Hydrophobic motif
Kog1	Kontroller of growth
Lst8	Lethal with SEC13 protein 8
MS	Mass spectrometry
mTOR	Mammalian [or mechanistic] target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
ORF	Open reading frame
PDK1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology, domain

PI3K	Phosphatidylinositol 3-kinase
PIKK	Phosphatidylinositol 3-kinase-related kinases
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich Akt/protein kinase B [PKB] substrate 40 kDa
PRR5	Proline-rich protein 5 [also called Protor]
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
raptor	Regulatory associated protein of mTOR complex 1
RBD	Ras binding domain
Rheb	Ras homolog enriched in brain
rictor	Rapamycin insensitive companion of mTOR
RNC	raptor N-terminal conserved
RP	Ribosomal protein
S6K	Ribosomal protein S6 kinase
SEA	Seh1-associated
SEAC	SEA complex
SEACIT	SEAC subcomplex inhibiting TORC1 signaling
SEACAT	SEAC subcomplex activating TORC1 signaling
Sin1	SAPK [stress-activated protein kinase]-interacting protein 1
SMG1	Serine/threonine-protein kinase SMG1
Tap42	Two A phosphatase associated protein
Tip41	Tap42 interacting protein
TCA	Tricarboxylic acid cycle or citric acid cycle
Tco89	TOR complex one 89 KDa subunit
TOR	Target of rapamycin
TORC1	TOR complex 1
TORC2	TOR complex 2
TOS	TOR signaling motif
TRRAP	Transformation/transcription domain-associated protein
TSC	Tuberous sclerosis complex

1 INTRODUCTION

1.1 TOR DISCOVERY AND EARLY STEPS

1.1.1 Rapamycin and TOR

The history of TOR starts with the discovery of rapamycin in 1965. Rapamycin is a macrolide isolated from a culture of *Streptomyces hygroscopicus* found in a soil sample from Easter Island (also known as Rapa Nui, therefore the name rapamycin). Rapamycin was initially identified for its ability to inhibit growth of the fungus *Candida albicans*, while it showed no inhibitory effect on the growth of gram-positive or gram-negative bacteria (Sehgal et al., 1975; Vézina et al., 1975). Later on, rapamycin immunosuppressive and cytostatic properties in higher eukaryotes were uncovered (Calne et al., 1989; Collier, 1989; Thomson and Woo, 1989; Tocci et al., 1989). Therefore rapamycin had to target a cellular component involved in cell growth and conserved among eukaryotes.

For more than 20 years rapamycin remained an orphan drug. Only in 1991 the target of rapamycin (TOR) was identified in a genetic screen in the budding yeast *Saccharomyces cerevisiae* (Heitman et al., 1991). The rapamycin resistant mutants found in the genetic screen led to the isolation of three genes: *FPR1*, *TOR1* and *TOR2*. *FPR1* encodes for FKB1, a peptidyl-prolyl cis-trans isomerase. *FKB1* is the yeast homolog of FKBP12, highly conserved among eukaryotes. *TOR1* and *TOR2* encode for two related Ser/Thr protein kinases that share 67% of identity. Yeast *TOR1* and *TOR2* probably arose from an ancient *Saccharomyces* genus-specific whole genome duplication, since only one TOR gene is found in higher eukaryotes (Wolfe and Shields, 1997). TOR1 and TOR2 are the founder members of the phosphatidylinositol 3-kinase-related kinases (PIKK) family. This class of kinases shares similarities with lipid kinases, but members of the PIKK class were shown to be not active towards lipids.

To inhibit TOR activity, rapamycin has first to form a complex with FKBP12. This mechanism of inhibition is analogous to inhibition of calcineurin by FKBP12 in complex with FK506, an immunosuppressive compound similar in structure to rapamycin (Schreiber, 1991). In the absence of FKB12, rapamycin no longer inhibits TOR. Organisms such as *Arabidopsis thaliana* and *Caenorhabditis elegans*, which lack FKBP12, are rapamycin resistant.

Thus, mutations in *FPR1* affecting rapamycin binding, and mutations in the rapamycin-FKBP12 binding site of *TOR1* and *TOR2* (Ser1972Arg and Ser1975Ile) could explain the rapamycin resistance strains found in the original genetic screen by Heitman and colleagues.

Genetic evidence collected in the early years of TOR discovery suggested the existence of two discrete TOR signaling branches: one rapamycin-sensitive, the other rapamycin-insensitive. This conclusion is based on the evidence that *TOR1* and *TOR2* deletion mutants displayed different phenotypes, despite the high identity shared at the gene level. Disruption of *TOR1* slightly reduces cell growth, while *TOR2* deletion is lethal and not rescued by *TOR1* overexpression. Cells depleted of *TOR2* arrest randomly in the cell cycle with a depolarized actin cytoskeleton, while rapamycin treated cells or cells lacking both *TOR1* and *TOR2* arrest in G1 phase as unbudded cells. Thus, TOR appears to exert two different functions. The first function is rapamycin-sensitive, redundant between *TOR1* and *TOR2* and required for G1 progression. The second function is rapamycin-insensitive, unique for *TOR2* and required for actin cytoskeleton dynamics (Cafferkey et al., 1993; Helliwell et al., 1998a; Kunz et al., 1993; Zheng et al., 1995). The elucidation of TOR domains and their function was essential for understanding the regulation of the two distinct TOR signaling branches.

1.1.2 From TOR domains to TOR complexes

TOR is a large kinase of 300 KDa circa. In the TOR N-terminal region are found several HEAT repeats (Huntingtin, EF3, PP2A and the yeast kinase TOR1). HEAT repeats are units of 40-50 amino acids each, forming antiparallel α-helices. In general, tandem HEAT repeats form a very flexible superhelical structure required for protein-protein interaction (Andrade and Bork, 1995; Groves et al., 1999). In the C-terminal part of TOR, conserved among the other PIKK members (ATM, ATR, DNA-PK, SMG1, and TRRAP) are found several other domains. In order from N- to C-terminus are the FAT, FRB, kinase, FIT and FATC domains. The FAT (FRAP, ATM and TRRAP) domain, assembled from multiple HEAT repeats, is circa 600 amino acids long. Following the FAT domain is the FRB domain (FKBP12-rapamycin binding domain), important for the binding of the rapamycin-FKBP12 complex (Choi et al., 1996) and unique for TOR among the PIKK members. The FRB domain (100 amino acids) contains the original rare missense mutations conferring rapamycin resistance to *TOR1-1* and *TOR2-1* mutants (Heitman et al., 1991). Immediately C-terminal to the FRB domain is the TOR kinase domain (300 amino acids). After the kinase domain is the circa 100 residues long FIT domain (Found in TOR), not highly conserved in other species. The FIT

domain is spatially close to the kinase active site, subjected to phosphorylation, and may act as repressor domain (Banaszynski et al., 2005; Chiang and Abraham, 2005; Holz and Blenis, 2005; Peterson et al., 2000; Sturgill and Hall, 2009). At the extreme C-terminus is the FATC domain (FAT C-terminal). Even though only 33 amino acids long, this domain is essential for TOR activity (Dames et al., 2005).

Early genetic studies in budding yeast predicted that the two TOR signaling branches could be dependent on the non-interchangeable HEAT domains of TOR1 and TOR2 and not on the interchangeable kinase domains (Helliwell et al., 1994). A breakthrough discovery in the TOR field came with the biochemical characterization of two distinct TOR multiprotein complexes. These two complexes, termed TOR complex 1 (TORC1) and TORC2, are essential and highly conserved in eukaryotes (Loewith et al., 2002). Indeed, TOR HEAT repeats resulted indispensable in TOR complexes formation, biochemically confirming the early genetic evidence.

The rapamycin sensitive TORC1 mediates temporal growth of the cell in response to nutrients. TORC1 positively regulates anabolic processes such as translation initiation, transcription, ribosome biogenesis, nutrient uptake and cell size. At the same time, TORC1 negatively regulates catabolic processes such as autophagy and ubiquitin-mediated proteolysis, and down-regulates stress response. TORC2 is rapamycin insensitive and controls the spatial growth of the cell by regulating actin cytoskeleton dynamics, cell wall integrity pathway, and sphingolipid biosynthesis. The nutritional cue associated with TORC2 activation is still elusive. In mammals, mTORC1 and mTORC2 are both activated by growth factors in a PI3K dependent manner. Additionally, mTORC1 responds to amino acid availability, cellular energy and oxygen levels. Thus, TOR couples growth to nutrient availability in eukaryotes.

Here the focus is on TOR signaling in budding yeast and to some extent on the parallelisms existing between yeast and mammalian cells. Following is a list of exhausting reviews about TOR signaling in yeast and other model organisms.

- (Jacinto and Hall, 2003) TOR in yeast, mammalian and flies
- (De Virgilio and Loewith, 2006) TOR in budding yeast
- (Blagosklonny and Hall, 2009) TOR and aging
- (Grewal, 2009) TOR in flies
- (Soulard et al., 2009) TOR in invertebrates
- (Hall and Tamanoi, 2010) TOR signaling
- (John et al., 2011) TOR in plants
- (Loewith and Hall, 2011) TOR history
- (Wei and Zheng, 2011) TOR and longevity

- (Robaglia et al., 2012) TOR in plants
- (Laplante and Sabatini, 2012) Mammalian TOR signaling

1.1.3 TORCs composition and localization

The discovery of the two TOR complexes finally explained TOR signaling branching. Two discrete TOR complexes exist, each one with its essential function and exclusive binding partners (Loewith et al., 2002). The core components of TORC1, in budding yeast, are TOR1 or TOR2, Lst8 and Kog1. TORC2 core components are TOR2, Lst8, Avo1 and Avo3. The finding that TOR2 could substitute for TOR1 in the TORC1 context helped explaining the different phenotypes associated with TOR genes deletions. In fact, *TOR2* is an essential gene, while *TOR1* is not. Therefore, in the absence of TOR1, a still functional TORC1 containing TOR2 supports cell growth. On the other hand, when TOR2 is depleted, TORC2 essential function is missing, given that TOR1 cannot substitute for TOR2 in TORC2. Exclusive binding partners confer specificity to the TOR complexes.

TOR complex 1

Kog1 (kontroller of growth) is the homolog of raptor in mammals. Kog1 has a molecular weight of 176 KDa and contains an N-terminal RNC domain (raptor N-terminal conserved), four internal HEAT repeats and seven C-terminal WD-40 repeats. Kog1 and raptor are required for TORC1 activity (Loewith et al., 2002; Wedaman et al., 2003), and play a positive role in TOR signaling by functioning as scaffold protein to recruit substrates to TORC1 (Kim et al., 2002; Nojima et al., 2003; Schalm et al., 2003; Yonezawa et al., 2004). Substrates of mammalian TORC1 (mTORC1) such as S6K and 4E-BP1 contain a TOS motif (TOR signaling) recognized by the RNC domain of raptor, greatly enhancing TOR-mediated substrate phosphorylation. Additionally, raptor acts to stabilize TOR and confers the capacity to respond to upstream signals (Kim and Sabatini, 2004). From a low resolution electron microscopy of the TOR-Kog1 complex, Kog1 was shown to interact with the N-terminal HEAT containing region of TOR, and with some other regions encompassing parts of the helical repeats and FAT domain, and the FAT, FRB, and kinase domain (Adami et al., 2007).

Lst8 (yeast lethal with sec thirteen) is a 34 KDa Gβ-like propeller protein structurally defined by seven WD-40 repeats. Lst8 binds to the kinase domain of both TOR complexes (Loewith et al., 2002; Wullschleger et al., 2005). In yeast Lst8 is required for integrity and activity of TORC2. Mammalian Lst8 (mLST8) strongly stimulates the catalytic activity of mTOR, but it appears to be more important for TORC2 signaling (Guertin et al., 2006; Kim et al., 2003). Another non-conserved protein found in TORC1 is Tco89 (TOR complex one 89 KDa subunit) (Reinke et al., 2004). Following rapamycin treatment, all

components of TORC1 can be co-precipitated with FKBP12. Thus, contrary to mammalian TORC1 (Yip et al., 2010), the structural integrity of yeast TORC1 is not compromised by rapamycin.

TOR complex 2

Avo1 and Avo3 (adheres voraciously to TOR2) are both essential proteins and exclusive binding partners of TORC2. Both proteins bind to the N-terminal HEAT repeats of TOR2 and are required for complex stability and function. Avo1 is required for Avo3 binding to TOR2, but not for TOR2 binding to Lst8. Avo1 is thought to mask the FRB site of TOR2 when TORC2 is assembled, explaining TORC2 insensitivity to rapamycin (Wullschleger et al., 2005). Moreover, Avo1 contains a C-terminal PH-like domain (pleckstrin homology) required for TORC2 membrane localization (Berchtold and Walther, 2009). The mammalian homologues of Avo3 and Avo1 are rictor and mSin1 respectively. As for Avo3, rictor specific function in mTORC2 is unknown. mSin1 undergoes alternative splicing. Five mSin1 splice variants are known (mSin1.1-5). Only TORC2 containing mSin1.1 and mSin1.2 is stimulated by insulin. A TORC2 containing mSin1.5 is insulin resistant (Frias et al., 2006). Notably, mSin1.5 is the only isoform lacking the PH and the Raf-like Ras-binding (RBD) domains. Both PH and RBD domains target mSin1 to membranes. These findings imply that insulin stimulation of TORC2 requires a membrane bound TORC2. Other non-conserved proteins found in yeast TORC2 are Avo2 and Bit61 (Binding partner of TOR2).

PRAS40, Deptor, PRR5 and PRR5L are components of the TOR complexes found only in mammals. PRAS40 is found only in mTORC1 and was shown to negatively regulate mTORC1 activity (Sancak et al., 2007; Vander Haar et al., 2007). Deptor binds to both complexes and regulates positively and negatively mTORC1 activity (Peterson et al., 2009). Deptor is in turn regulated at the translational level by both mTORC1 and mTORC2. PRR5 and PRR5L are found in mTORC2 where they bind rictor. Both are not required for rictor and mSin1 binding to mTOR.

TOR complexes dimerization and localization

Both complexes are thought to exist as multimers, likely as homo-dimers (TORC1-TORC1 and TORC2-TORC2). Dimerization is mediated by HEAT-FAT interactions between TOR proteins (Loewith et al., 2002; Takahara et al., 2006; Wang et al., 2006; Wullschleger et al., 2005; Zhang et al., 2006). In yeast, TOR dimerization is not influenced by nutrients, while in mammalian cells presence of amino acids stimulates mTOR dimerization. Rapamycin and

growth factors do not affect multimerization events. Thus, it is not yet clear whether TOR dimerization reflects kinase activation.

The cellular localization of the two complexes is different, consistent with the two essential distinct functions carried out by TORC1 and TORC2. Yeast TORC1 localizes at the vacuolar membrane (Berchtold and Walther, 2009; Binda et al., 2009; Chen and Kaiser, 2003; Reinke et al., 2004; Sturgill et al., 2008; Urban et al., 2007). At this location TORC1 is catalytically active. Rapamycin or nutrient starvation does not alter TORC1 localization. In yeast, the vacuole is the storage compartment for nutrients. Thus, TORC1 vacuolar localization might be linked to its ability to sense nutrients. Others reported a nucleolar localization for a fraction of TORC1, where it actively controls 35S rRNA transcription (Li et al., 2006). mTORC1 localizes on endosomal membranes upon nutrient stimulation and is diffuse in the cytoplasm during starvation (Long et al., 2005b; Sancak et al., 2008).

Yeast TORC2 is localized at or near the plasma membrane (Aronova et al., 2007; Kunz et al., 2000; Sturgill et al., 2008; Wedaman et al., 2003) where it defines a TORC2-specific domain (Berchtold and Walther, 2009). The PH domains of Avo1 mediate the anchoring of TORC2 to the plasma membrane. Also mTORC2 localizes at the plasma membrane, and at the interface between ER and mitochondria (MAM) (Betz et al., 2013).

1.1.4 Perspective in TOR signaling research

Following the initial discovery of TOR, many efforts were made in understanding the role played by TOR in controlling cell growth. TOR signaling was (and still is) studied in different model organisms such as yeast, slime mold, plants, worms, flies, mammalian tissue culture cells and mice. In general TOR is activated by nutrients and growth factors, and inhibited by noxious stresses. In yeast several line of evidence suggest that TORC1 is activated in response to the availability of nutrients, particularly of the nitrogen source (amino acid and other nitrogenous compounds). Hence, TOR signaling ensures proper growth only in conditions that allow growth itself. In other words, TOR couples growth to nutrient availability. Proper control of growth required a further developmental step with the event of multicellularity in higher eukaryotes. Thus, another dimension of control acting at the tissue and whole organism level was required in addition to the "simple" single cell level. Therefore, growth factors signaling, which coordinate events affecting multiple tissues, engrafted on mTOR nutrient sensing and regulation. Importantly, deregulation of mTOR signaling leads to uncontrolled growth and metabolic homeostasis, leading to diabetes and cancer onset. Altered mTOR signaling is one of the most common hallmarks in human cancers. Rapamycin and rapamycin derivatives (rapalogs) were proven unsuccessful in cancer treatment. Thus, knowing the exact molecular mechanisms controlling mTOR activation and signaling might lead to development of promising new anticancer drugs. In the next sections the focus is on the upstream and downstream regulation of TOR complexes. Following is a list of reviews treating mTOR signaling in cancer and disease.

- (Benjamin et al., 2011) mTOR inhibitors
- (Dazert and Hall, 2011) mTOR and disease
- (Cantor and Sabatini, 2012) Cancer metabolism
- (Alayev and Holz, 2013) mTOR and cancer
- (Lamming et al., 2013) mTOR and aging
- (Vinayak and Carlson, 2013) mTOR and breast cancer
- (Zhang et al., 2013) Cancer metabolism

1.3 TORC2 SIGNALING

1.3.1 Downstream targets of TORC2

The impossibility to directly inhibit TORC2 with specific drugs left no choice to researchers to investigate TORC2 signaling by genetic means. Over the years many downstream functions of TORC2 were uncovered in this way. TOR2 (and later on TORC2) was shown to regulate cytoskeleton organization, cell wall integrity pathway and sphingolipids biosynthesis.

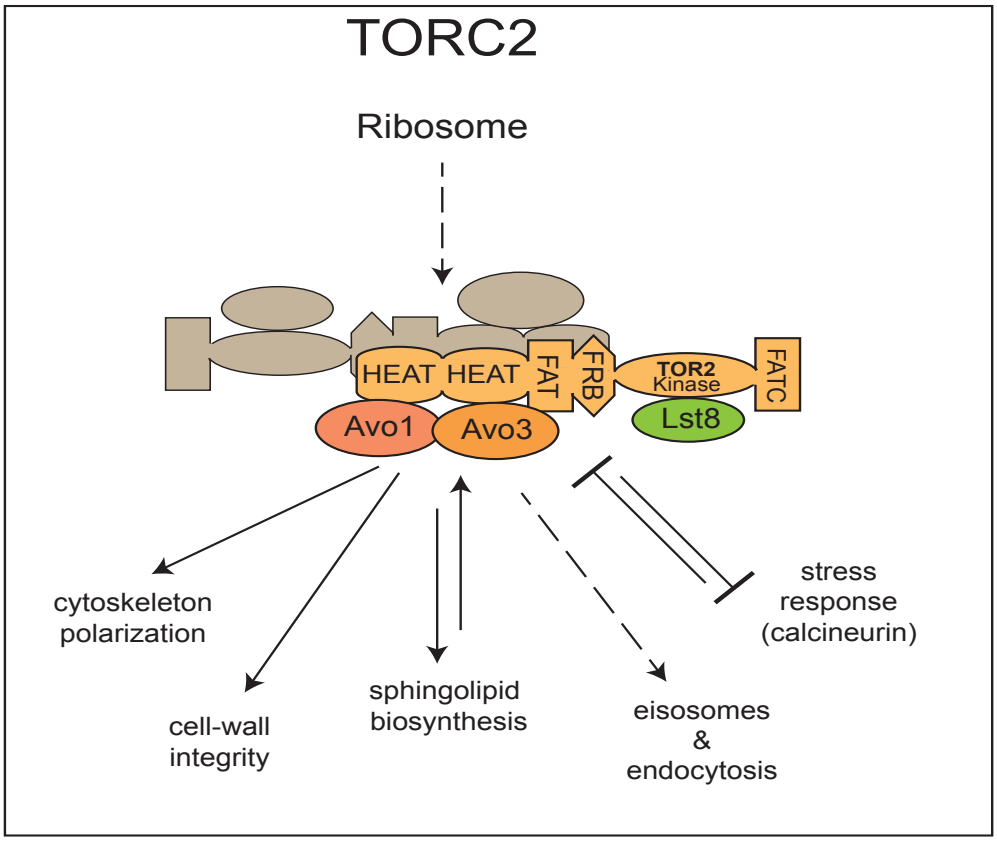

Figure 1: TORC2 signaling

Cytoskeleton organization

One of the earliest observations made on *tor2* mutants was that the cytoskeleton organization in these cells was compromised (Schmidt et al., 1996). Budding yeast growth is polarized towards the bud (daughter cell). In a cell cycle dependent way, from late G1 until mid G2 phase, the actin cables and patches are polarized to ensure transport of nutrients and organelles from the mother to the daughter cell. TOR2 control over cytoskeleton organization was later shown to be dependent on the activation of the small GTPase Rho1 (Schmidt et al., 1997). Deletion of *SAC7*, encoding for Rho1 GAP, suppressed the growth defect of *tor2*. Consistently, overexpression of *RHO1, RHO2*, and of the GEF for Rho1 *ROM2*, restored the growth defect of *tor2* cells. Thus, TOR2 control over Rho1 is mediated by activation of Rom2. The molecular mechanism at the basis of Rom2 activation by TOR2 is still unknown.

Cell wall integrity pathway

Further genetic studies showed that *PKC1* was another downstream effector of Rho1 able to restore the growth defect of a *tor2* mutant (Helliwell et al., 1998b). PKC1 controls a mitogen-activated protein kinase (MAPK) cascade pathway called the cell wall integrity (CWI) pathway. CWI is activated in response to stresses leading to the up-regulation of genes involved in cell wall biosynthesis (Heinisch et al., 1999). Similar to *PKC1*, overexpression of CWI component pathway such as *BCK1*, *MKK2* and *MPK1* restored the actin cytoskeleton depolarization of *tor2* mutants. Therefore, TOR2 via Rom2-dependent activation of Rho1 and PKC1 controls cytoskeleton polarization and CWI.

Sphingolipid biosynthesis

Sphingolipids are essential components of eukaryotic membranes. They are involved in actin cytoskeleton dynamics, endocytosis and membrane rafts organization. Sphingolipids biosynthesis steps take place at the ER and are well conserved among eukaryotes. Complex sphingolipids biosynthesis requires the production of relevant intermediates such as long-chain bases (LCB) and ceramides. Ceramides are then transported to the Golgi where they are further modified to complex sphingolipids. One of the final steps in ceramide biosynthesis, catalyzed by Csg1 or Csg2, converts inositol phosphoceramide (IPC) to mannose inositol phosphoceramide (MIPC). *csg2* mutants were shown to accumulate IPC and be hypersensitive (lethal) at low

calcium doses (Beeler et al., 1994). In the subsequent genetic screen aimed to uncover thermo-sensitive suppressors of *CSG2* deletion (TSC) *TOR2* and *TSC11* were identified (Beeler et al., 1998). *TSC11*, also known as *AVO3*, was later shown being an essential component of TORC2 (Loewith et al., 2002). These early genetic data implied an involvement for TORC2 in sphingolipid biosynthesis.

MSS4 was another gene identified in the *csg2* suppressor screen along with *TOR2* and *AVO3*. *MSS4* encodes for the yeast PI4,5-kinase, which catalyzes the formation of $PI4,5P_2$ from PI4P. Proteins possessing a PH domain can bind $PI4,5P_2$ and localize in this way to membranes. Some of these proteins in turn are involved in actin cytoskeleton, CWI and endocytosis regulation. Another genetic screen aiming to the identification of synthetic lethal genes in combination with a hypomorphic temperature-sensitive allele of *MSS4*, identified several genes. Among these genes was *AVO2*, component of TORC2 (Audhya et al., 2004). Other synthetic lethal with *MSS4* (SLM) genes were identified, such as *SLM1* and *SLM2*. Slm1/2 share overlapping functions and were shown to be direct TORC2 targets in two independent studies (Audhya et al., 2004; Fadri et al., 2005). Slm1/2 were lately involved in sphingolipids biosynthesis given that Slm1/2 mutants showed negative genetic interactions with component of the sphingolipids biosynthetic pathway and were hypersensitive to myriocin, a drug that inhibits the first step in sphingolipids biosynthesis (Daquinag et al., 2007).

The identification of other TORC2 downstream targets, the AGC kinases Ypk1 and Ypk2, helped in the further characterization of TORC2 involvement in sphingolipids biosynthesis. Ypk1/2 are phosphorylated by Pkh1 (yeast PDK1) in response to increasing levels of LCBs and further phosphorylated and activated by TORC2 (acting as PDK2) (Aronova et al., 2008; Kamada et al., 2005; Mulet et al., 2006). Overexpression of the hyperactive allele of *YPK2* (*YPK2*^{D239A}) is the only known suppressor of the lethality induced by *TOR2* loss. *YPK2*^{D239A} also restores the levels of ceramide which are drastically decreased when TORC2 is compromised (Aronova et al., 2008). Therefore, TORC2 positively regulates de novo ceramide biosynthesis via Ypk1/2.

A connection between TORC2, Slm1/2 and Ypk1/2 comes from a recent paper in which the author demonstrated that Ypk1 recruitment and activation by TORC2 is dependent on Slm1/2 which act as scaffold protein through their PH domain (Niles et al., 2012). Another publication showed that upon plasma membrane mechanical stress, Slm1/2 relocalized to the plasma membrane to activate TORC2 and Ypk1 to promote sphingolipids biosynthesis (Berchtold et al., 2012). This publication is more debatable since it inverts the classical role for TORC2 upstream of Slm1/2 activation.

More recently another set of proteins was shown to be important for sphingolipid biosynthesis. ORM are conserved ER membrane proteins which bind to and inhibit the first enzyme in sphingolipid biosynthesis, the serine-palmytoyl-coenzyme A transferase (SPT) (Han et al., 2010). Orm1/2 are inactivated by phosphorylation in response to compromised sphingolipid synthesis, leading to SPT activation restoring sphingolipids production (Breslow et al., 2010; Sun et al., 2012). TORC2 was shown to regulate Orm phosphorylation via Ypk1. Reduction in sphingolipids stimulated Ypk1 via TORC2-dependent phosphorylation. Ypk1 then phosphorylated Orm1/2 to restore sphingolipids biosynthesis. Thus, Ypk1 is both a sensor and effector of sphingolipid levels (Roelants et al., 2011).

Calcineurin signaling

A complex interplay exists between TORC2 and calcineurin signaling. Calcineurin is a conserved Ca^{2+}/calmodulin Ser/Thr protein phosphatase activated following stress conditions that increase the cytoplasmic concentration of Ca^{2+}. Active calcineurin dephosphorylates several targets, one of which is the transcription factor Crz1. Dephosphorylated Crz1 enters the nucleus and drives the transcription of several genes involved in survival following stress (Matheos et al., 1997).

TORC2 negatively regulates calcineurin signaling and Crz1 transcription, probably by modulating the interaction of Slm1/2 with the catalytic subunits of calcineurin. In this way, TORC2 suppresses stress response during optimal growth conditions (Mulet et al., 2006). Conversely, calcineurin interacts and dephosphorylates Slm1/2 leading to stress-induced changes in actin polarization and nutrient-regulated permease endocytosis and turnover. Therefore, calcineurin prevents TOR-mediated growth during stress conditions (Bultynck et al., 2006).

Eisosome assembly and disassembly

Eisosomes are the endocytosis entry sites at the plasma membrane. Pkh1/2, Ypk1/2 and LCB levels were involved in eisosome assembly and disassembly (Luo et al., 2008; Walther et al., 2007; 2006). Whether TORC2 is also directly involved in the regulation of eisosome formation and endocytosis remains to be confirmed. TORC2 localization at plasma membrane foci adjacent to eisosomes suggests for a direct involvement of TORC2 in endocytosis (Sturgill et al., 2008). On the other hand, TORC2 was shown to localize to a defined plasma membrane domain distinct to eisosomes (Berchtold and Walther,

2009). Thus, TORC2 involvement in eisosome formation and endocytosis has to be further investigated.

Overlap between TORC1 and TORC2 signaling

The strict division of substrates and function between the two TOR complexes, apparently so clear during the early genetic studies, started to be looser in the last years. Accumulated evidence suggests that a role for TORC1 in TORC2 signaling has to be considered. More specifically, rapamycin treatment was shown to trigger actin depolarization (Aronova et al., 2007). CWI is affected by deletion of non-essential components of TORC1 and mutations in the Tap42-phosphatase complex (Angeles de la Torre-Ruiz et al., 2002; Reinke et al., 2004; 2006; Torres et al., 2002; Wang and Jiang, 2003). Finally, TORC1 was shown to control ORM phosphorylation, and consequently sphingolipid biosynthesis, independently of TORC2, as a part of an adaptive response to nutrient stress. The mechanism involves activation of Npr1 when TORC1 is inhibited by rapamycin, leading to increase de novo synthesis specifically of complex sphingolipids (Shimobayashi et al., 2013).

1.3.2 TORC2 upstream signaling

Both in yeast and higher eukaryotes TORC2 activity is not stimulated by nutrients. In higher eukaryotes growth factors, particularly insulin, stimulate in a PI3K dependent way mTORC2 activity, leading to phosphorylation of mTORC2 targets such as Akt, SGK1 (the homolog of Ypk2), PKC, Rho1 and Rac1 to drive metabolism, survival, actin organization and cytoskeleton dynamics. mTORC2 activation by amino acids is controversial (Hernández-Negrete et al., 2007; Nobukuni et al., 2005; Tato et al., 2011).

Recent finding enabled a better understanding of the upstream regulation of TORC2. Once more, yeast genetics resulted essential in the process. An elegant yeast reverse suppressor screen identified *NIP7* as upstream regulator of TORC2. The genetic screen was based on the fact that mutants defective in TORC2 signaling require overexpressed hyperactive *YPK2* for survival. Following random mutagenesis, those mutants unable to lose a high copy number plasmid harboring *YPK2*^{D239A} were most probably defective in TORC2 signaling and potentially defective in upstream regulators of TORC2. Among genes encoding for core components of TORC2 such as *TOR2*, *AVO1* and *AVO3*, a gene involved in 60S ribosome maturation, *NIP7*, was discovered. The thermo-sensitive mutant identified, *nip7-1*, perfectly phenocopied loss of *TOR2* at non-permissive temperature, including actin depolarization, failure to induce CWI pathway, impaired sphingolipids biosynthesis, restoration of growth of a *csg2* strain in the presence of Ca^{2+}, decreased TORC2 and Ypk2 kinase activity (Zinzalla et al., 2011). Nip7 is conserved among eukaryotes and is required for ribosome biogenesis also in mammalian cells. Further characterization led to the discovery that the growth factor stimulated physical association of mTORC2 with the ribosome, independently of translation, is required for mTORC2 activation. Moreover, mTORC2-ribosome association, mediating PI3K-mTORC2-Akt signaling and cell survival, was functionally important in cancer cells. If TORC2-ribosome physical association is conserved also in yeast, and how the ribosome stimulates TORC2 activity are still unanswered questions.

In a follow-up study aimed to better understand the physiology of mTORC2-ribosome association, an essential role for mTORC2 in mitochondria-associated endoplasmic reticulum membranes (MAM) was uncovered. mTORC2 was shown to localize at MAM in a PI3K and ribosome association dependent manner. MAM-associated mTORC2 activates Akt and thereby controls MAM integrity, mitochondrial metabolism, and cell survival (Betz et al., 2013).

1.3 TORC1 SIGNALING

1.3.1 Downstream targets of TORC1

The possibility to chemically inhibit TORC1 signaling with rapamycin helped researchers to uncover the many cellular processes regulated by TORC1. In this section the focus is on TORC1 downstream targets, divided in proximal effectors and distal readouts.

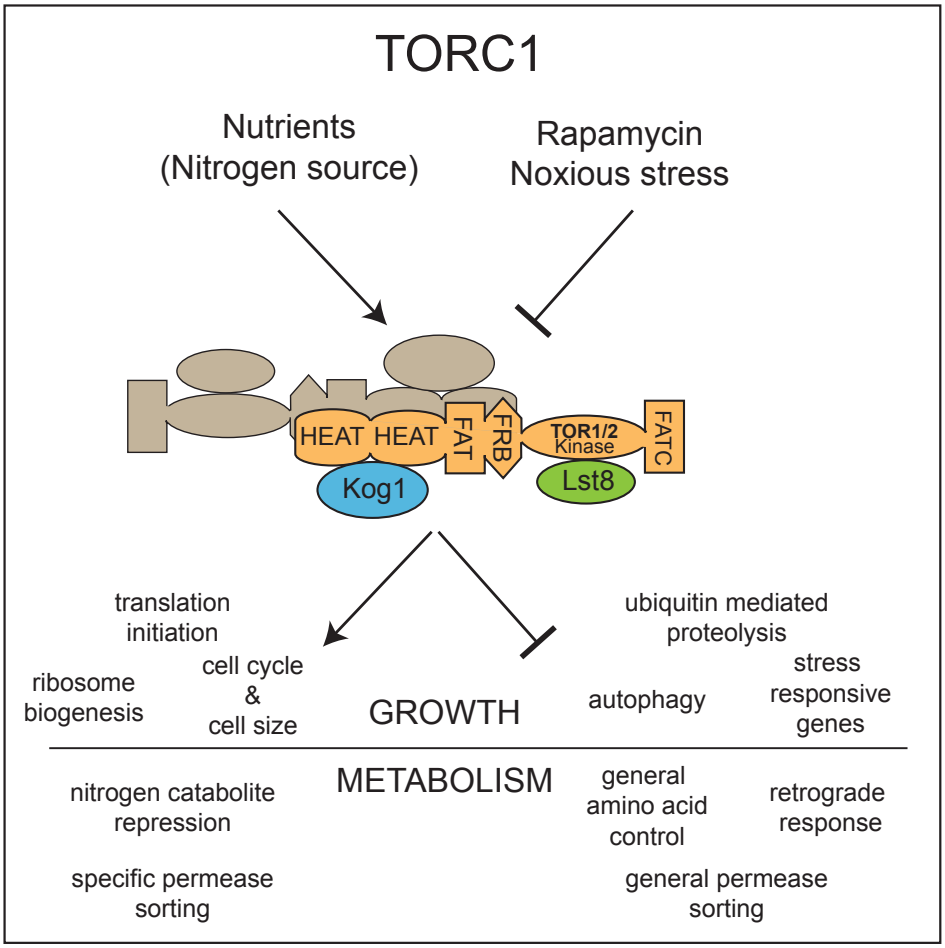

Figure 2: TORC1 signaling

PROXIMAL EFFECTORS OF TORC1 SIGNALING

A clear bifurcation in TORC1 signaling was highlighted by phosphoproteomic studies in response to rapamycin treatment (Huber et al., 2009). The protein kinase Sch9 on one side and the Tap42/phosphatase complex on the other, mediate these two effector branches. Many proteins exhibited phosphorylation changes dependent only on one or the other activity, highlighting the independence of the two downstream pathways. Some rapamycin-induced changes in phosphorylation occurred were dependent on both Sch9 and Tap42. More interestingly, some other phosphorylation events happened independently of Sch9 and Tap42, suggesting the existence of other TORC1 downstream pathways.

The AGC kinase Sch9

The AGC kinase Sch9, homolog to mammalian S6 kinase and Akt, is probably the best-characterized direct substrate of TORC1. As all AGC kinases, Sch9 activity is stimulated by phosphorylation events (Arencibia et al., 2013; Jacinto and Lorberg, 2008; Pearce et al., 2010). Phosphorylation on the activation- or T-loop by PDK1 is required for Sch9 activity. The yeast PDK1 is Pkh1, a kinase positively regulated by sphingolipid levels. Further phosphorylation events, thought to enhance or confer specificity to the AGC kinase activity, take place in the hydrophobic (HM) and turn motif (TM). Phosphorylation at these sites is dependent on PDK2. In the past years, mTOR importance as regulator of mammalian AGC kinases has emerged (Jacinto and Lorberg, 2008). mTOR, in the mTORC2 context, phosphorylates Akt Ser473 within the hydrophoibic motif of Akt. Thus, mTORC2 acts as PDK2 for Akt. The situation is similar in yeast, where it was shown that TORC1 phosphorylates Sch9 on at least six residues. All these residues are localized on Sch9 C-terminus, within Sch9 HM, TM and HM-like motives. TORC1-dependent phosphorylation of at least five of the six sites was required for Sch9 activity in vitro and in vivo (Urban et al., 2007).

Sch9 is also regulated by the carbon source, in addition to regulation by the nitrogen source via TORC1. Sch9 levels and phosphorylation were regulated by glucose (Jorgensen et al., 2004). Interestingly, Sch9 was originally discovered as multicopy-suppressor in a strain lacking PKA activity (Toda et al., 1988). Sch9 overexpression resulted in the up-regulation of several genes, which were also targets of PKA. PKA is activated in response to the carbon source. In the absence of PKA, a part of the glucose induced transcriptional response is due to Sch9 activity (Zaman et al., 2009). Thus, it appears that both carbon (even though only partially) and nitrogen source

converge on Sch9 regulation. Processes downstream of TORC1 regulated by Sch9 are: translation initiation, transcriptional regulation of ribosomal protein (RP) and ribosome biogenesis factors (RiBi), entry into G0 phase and regulation of G1 progression, transactivation of stress-responsive genes, lifespan regulation.

The protein phosphatase 2A (PP2A)

The other effector branch downstream of TORC1 involves protein phosphatases. Particularly, TORC1 regulates type 2A (Pph21, Pph22 and Pph3) and 2A-related phosphatases (Sit4 and Ppg1). TORC1 regulation of these phosphatases requires the regulatory subunit Tap42. TORC1 regulates directly or indirectly via Tip41, the phosphorylation state of Tap42 (Jacinto et al., 2001; Jiang and Broach, 1999). Phosphorylated Tap42 forms tight complexes with the phosphatases (Di Como and Arndt, 1996) and the peptidyl-prolyl cis/trans isomerases Rrd1 and Rrd2 (Zheng and Jiang, 2005). The Tap42-phosphatase complex is thought to inactivate or redirect towards different substrates the phosphatase activity (Düvel et al., 2003; Yan et al., 2006). Upon starvation or treatment with rapamycin the Tap42-phosphatase complexes are released and result in phosphatase activation or change in substrate preference, and Tap42 dephosphorylation over time. Downstream of TORC1, PP2A regulates translation initiation, and the dephosphorylation of Gln3 and Gat1, two transcription factors controlling the nitrogen catabolite repression (NCR).

Other downstream targets

Several other downstream targets of TORC1 were identified over the years. Other TORC1 direct targets comprehend the transcriptional activators Gln3 and Sfp1, respectively involved in NCR and ribosome biogenesis regulation, and Atg13, a protein involved in autophagy. A recent study, focused on uncovering the global interaction network of protein kinase and phosphatase in yeast, described new potential TORC1 targets (Breitkreutz et al., 2010). The importance in TORC1 signaling of some of these newly identified substrates, such as Npr1 and Ksp1 was subsequently proven in more recent studies (Panchaud et al., 2013; Umekawa and Klionsky, 2012).

TORC1 DISTAL READOUTS

Translation initiation

Rapamycin treatment blocks protein translation leading to decreased protein synthesis and arrest in G1 phase of the cell cycle (Barbet et al., 1996). In both yeast and mammalian cells TORC1 controls cap-dependent mRNA translation.

In yeast, TOR promotes cap-dependent translation acting on the phosphorylation of the eIF2α kinase Gcn2 (Gallinetti et al., 2012; Murguía and Serrano, 2012). eIF2α is a component of the met-tRNAi ternary complex. eIF2α phosphorylation by Gcn2 inhibits eIF2B GEF activity toward eIF2α leading to the formation of the inactive eIF2α-P-GDP-eIF2B complex. The eIF2α-P-GDP-eIF2B complex, by sequestering eIF2α, compromises translation initiation. In the presence of unloaded tRNAs Gcn2 is activated and phosphorylates eIF2α. Gcn2 activity is also regulated by phosphorylation on Ser577. TORC1 promotes by two distinct mechanisms the phosphorylation of Ser577. First, TORC1 positively regulates Sch9, which probably in turn phosphorylates Ser577. Second, TORC1 negatively regulates PP2A preventing Ser577 dephosphorylation (Cherkasova, 2003).

In higher eukaryotes TOR promotes cap-dependent translation by directly phosphorylating 4E-BP1. 4E-BP1 competes for the binding to eIF4E with eIF4G. When 4E-BP1 is hyperphosphorylated it can no longer bind to eIF4E, which then is free to bind eIF4G and drive translation initiation.

Ribosome biogenesis

Nutrients fuel cell growth, a process that depends primarily on the biosynthetic capacity of the cell, in turn dependent on ribosomes. Circa 95% of total transcription in exponentially growing yeast is dedicated to ribosome biogenesis. Ribosome biogenesis requires not only the action of the three RNA polymerases but also protein synthesis itself. rRNA, tRNA, ribosomal proteins and ribosome assembly factors must be transcribed and translated to ensure proper ribosome biogenesis. Thus, ribosome biogenesis is very expensive in terms of energy and building blocks consumed. Therefore, ribosome biogenesis has to be down-regulated during times of nutrient scarcity, or cells would face energetic failure and certain death. Therefore, ribosome biogenesis is tightly linked to nutrient availability. TORC1 regulates ribosome biogenesis at the transcriptional and translational level by different mechanisms (Powers and Walter, 1999).

RNA Pol I and RNA Pol III transcripts (rRNA and tRNA) are controlled by TORC1 via protein synthesis. In fact, ribosomal RNA processing requires

the presence of ribosomal proteins to be incorporated into maturing ribosomes. Unprocessed rRNA is rapidly degraded (Warner, 1999). Thus, a decrease in free ribosomal proteins leads to rRNA degradation. Inhibiting protein translation, such as following rapamycin treatment, would produce a decrease in rRNA levels (Reiter et al., 2011; Tschochner and Hurt, 2003). As alternative mechanism, TORC1 was proposed to regulate RNA Pol I by affecting the polymerase loading at the rDNA locus (Huber et al., 2009). In the same study, it was proposed a role for Sch9 in regulating RNA Pol III transcription via Maf1 phopshorylation. Maf1 is a RNA Pol III repressor. When phosphorylated by Sch9, Maf1 binding and repressor activity to RNA Pol III is inhibited, ensuring proper tRNA transcription.

Rna Pol II transcripts (RP and RiBi genes) are promoted via three different TORC1-dependent mechanisms, involving Sch9, Sfp1 and Fhl1. Via Sch9, TORC1 ensures that the transcriptional repressors Dot6, Tod6 and Stb3 are hyperphosphorylated and thus excluded from the nucleus (Huber et al., 2011). TORC1 phosphorylation of Sfp1, allows Sfp1 to interact with the RP promoters and drive locus transcription (Lempiäinen et al., 2009). Sfp1 phosphorylation is intriguingly insensitive to nutrients or stress. Sfp1 overexpression also accounts for increased RiBi transcription (Jorgensen et al., 2004). Fhl1 is constitutively bound to RP genes promoters. TORC1 influences the binding of the co-activator Ifh1 or of the co-repressor Crf1 to Fhl1, determining RP transcription fate. When TORC1 is active Ifh1 is phosphorylated and can interact with Fhl1 activating RP transcription. Conversely, when TORC1 is inactive, Crf1 is phosphorylated and thus binds to Fhl1 inhibiting RP transcription (Berger et al., 2007; Lee et al., 2002; Martin et al., 2004; Rudra et al., 2005; Schawalder et al., 2004; Wade et al., 2004).

Cell cycle and cell size

Growth (mass accumulation) and cell cycle are distinct co-regulated mechanisms. Mitosis is an energy demanding process and mitotic commitment requires the availability of sufficient nutrients to ensure successful duplication. During the cell cycle, cells are committed to a new mitotic cycle following START. At START converge the inputs deriving from nutrient availability. A critical mass threshold has to be reached before passing START. The mechanism involved in attaining critical mass is still poorly understood. What is known is that the translational machinery, and thus protein synthesis, plays a major role. Possibly, there is a link between translation rate and accumulation of the G1 cyclin Cln3. Cln3 is very unstable and thus requires a robust translating machinery to accumulate and drive cell cycle progression (Barbet et al., 1996). Deletion of *SFP1* or *SCH9* by

decreasing ribosome biogenesis, has the effect to reduce the size required for passing START (Jorgensen et al., 2004). Therefore, TORC1 regulates and couples nutrient cues to cell cycle progression. Consistently, rapamycin treatment causes arrest in G1 phase by decreasing the levels of the G1 cyclins Cln1-3 (Barbet et al., 1996) and up-regulating the Cdk inhibitor Sic1 (Zinzalla et al., 2007). Another interesting observation is that rapamycin treatment causes a different phosphorylation status of the G1 transcription repressor Whi5 and the G1 transcription activator Msa1 (Ashe et al., 2008; Costanzo et al., 2004). Moreover, rapamycin treatment elicits a paradoxical increase in cell size due to increase autophagy in G1 arrested cells.

Independently of ribosome biogenesis TORC1 appears to control the G2/M transition (Nakashima et al., 2008). The authors showed that TORC1 regulated the polo-like kinase Cdc5 subcellular localization via the Tap42-phosphatase branch of TORC1. Cdc5 proper localization was required for phosphorylation and destabilization of Swe1, which negatively regulates Cdc28 kinase activity and G2/M progression.

Stress responsive genes and autophagy

Rapid response to environmental insults is essential for survival. Erroneous activation of stress response during growth-favoring conditions has negative effects on growth itself. TORC1 negatively regulates stress response during growth.

The environmental stress response (ESR) is elicited by a large number of environmental insults, including nutrient deprivation, heat, oxidative stress, and high osmolarity (Gasch et al., 2000). A large transcriptional response leading to the up-regulation of more than 300 genes is activated in response to ESR. The redundant Msn2 and Msn4 transcription factors bind to STRE elements to activate transcription of a large number of stress responsive genes. Nutrient availability impinges on Msn2/4 activity by modulating levels of phosphorylation of various sites in turn regulating Msn2/4 nuclear entry, nuclear exit, and transcriptional activation. Msn2/4 phosphorylation is regulated by PKA and TORC1. PKA phosphorylation of Msn2/4 promotes their nuclear export and blocks their nuclear import, preventing ESR genes transcription. In a similar fashion, TORC1 prevents Msn2/4 dephosphorylation by negatively regulating PP2A. Moreover, TORC1 negatively regulates Msn2/4 activity via the Sch9/Rim15 axis. Active Sch9 phosphorylates Rim15 favoring Rim15 interaction, and cytoplasmic anchoring, with 14-3-3 proteins (Wanke et al., 2008; 2005). Rapamycin treatment leads to Rim15 dephosphorylation, release from 14-3-3 and nuclear localization. Nuclear Rim15 up-regulates the expression of Msn2/4 regulated genes. Another effect

of Rim15 activation is to phosphorylate Igo1/2. Igo1/2 are α-endusolfine paralogs which bind and protect from degradation newly transcribed mRNAs. Igo1/2 activation by Rim15 is essential to prevent stress responsive genes mRNA degradation and thus, ensure proper translation and activation of ESR (Luo et al., 2011; Talarek et al., 2010).

Autophagy is an essential process that ensures availability of new building blocks via degradation of cytoplasmic proteins and organelles. Autophagy is especially important to ensure survival during nutrient limitation. Two mechanism of autophagy exist in yeast: macro- and micro-autophagy. Macro-autophagy describes the packaging of bulk cytoplasmic portions in a double membrane structure called autophagosome. In this way, cytoplasmic material and organelles such as peroxisomes or mitochrondria are delivered to the vacuole where they are degraded and recycled in amino acids and fatty acids. TORC1 regulates macro-autophagy by dictating the phosphorylation state of Atg13 (Kamada et al., 2010). Phosphorylated Atg13 prevents Atg1 kinase association in the Atg1-Atg17-Atg29-Atg31 sub-complex. Upon TORC1 inhibition, Atg13 is dephosphorylated and allows Atg1 recruitment to the complex. Once complexed, Atg1 is phosphorylated and activated leading to macro-autophagy onset. Atg1 activation is absolutely essential for macro-autophagy induction, as *atg1* mutants display no macro-autophagy. Moreover, dephosphorylation of Atg13 is sufficient to induce macro-autophagy because expression of a non-phosphorylatable version of Atg13 yields induction of autophagy in cells growing in rich medium (Kamada, 2010).

In addition to TORC1, also PKA negatively controls macro-autophagy. Simultaneous inactivation of PKA and Sch9 yields partial activation of the autophagic response without significantly altering the phosphorylation state of Atg13. Moreover, the effect on autophagy of PKA and Sch9 inactivation is synergistic with TORC1 inactivation, suggesting that PKA acts in parallel to TORC1. The dominant role of TORC1 over PKA in regulating autophagy may reflect the importance of autophagy in protecting cells from nitrogen starvation rather than from carbon starvation. This is based on the fact that the major product of macro-autophagy are amino acids and that mutants defective in autophagy are much more sensitive to nitrogen or phosphate starvation than to glucose starvation (Klosinska et al., 2011).

In micro-autophagy the cytoplasmic material is directly assimilated in the vacuole through invagination of the vacuolar membrane. TORC1 was also shown to negatively regulate micro-autophagy (Dubouloz et al., 2005). In this context micro-autophagy serves to restore cell growth following rapamycin treatment, at least in part by reducing the amount of vacuolar membrane that accumulates as a result of starvation-induced macro-autophagy.

1.3.2 TORC1 and nitrogen metabolism

The response of yeast cells to TOR depletion or rapamycin treatment mimics a nutrient stress response, including down-regulation of translation initiation, inhibition of ribosome biogenesis (down-regulation of rRNA and ribosomal protein transcription), cell cycle arrest in G1 phase, storage carbohydrate accumulation (glycogen), increase in amino acid permeases activity and autophagy (Barbet et al., 1996; Chan et al., 2001; Noda and Ohsumi, 1998; Powers and Walter, 1999; Schmidt et al., 1998; Zaragoza et al., 1998). In addition, genome-wide analysis of genes induced upon rapamycin treatment revealed that TOR controls nutrient responsive genes. Many of these genes are involved in the glycolytic pathway and the tricarboxylic acid (TCA) cycle, but the most striking set of genes affected by rapamycin treatment is composed of genes involved in the assimilation of different nitrogen sources (Cardenas et al., 1999; Hardwick et al., 1999; Shamji et al., 2000). Thus, of particular mention for this work is the relationship existing between the nitrogen source and TORC1 signaling. TORC1 is influenced by the nitrogen source, and in turn TORC1 controls the transcription of genes required for nitrogen source utilization. In the next section the focus is on the regulatory mechanisms that partially explain this synergy. Following is a list of useful reviews treating nitrogen metabolism and regulation in yeast.

- (Cooper, 1982) Nitrogen metabolism
- (Magasanik, 1992) Nitrogen utilization
- (Hofman-Bang, 1999) Nitrogen catabolite repression
- (Schure et al., 2000) Ammonia metabolism
- (Cooper, 2002) TOR and GATA factors
- (Magasanik and Kaiser, 2002) Nitrogen regulation
- (Butow and Avadhani, 2004) Retrograde response
- (Wong et al., 2008) Nitrogen regulation
- (Ljungdahl, 2009) SPS sensing pathway
- (Jazwinski and Kriete, 2012) Retrograde response
- (Ljungdahl and Daignan-Fornier, 2012) Nitrogen metabolism regulation

ON YEAST METABOLISM

The relation between nutrient availability, metabolism and cell proliferation is complex and far from being completely understood. Unicellular organisms, such as yeasts and bacteria, reproduce as quickly as nutrient availability allows them to, and enter a quiescent stationary state when nutritional conditions become adverse. Metabolic control systems have evolved to sense nutrient supply and channel the required carbon, nitrogen, and free energy into generating the building blocks needed for survival and replication. In general, in a rich medium yeast cells are larger and duplicate faster. Glucose presence in the media elicits the best-known control system over metabolism, intracellular signaling, growth and proliferation.

Glucose sustains faster growth compared to all other carbon sources. When glucose is provided, yeast cells adopt a fermentative metabolism that channels glucose through glycolysis for energy production, even in the presence of oxygen. The pyruvate produced via glycolysis is then converted to ethanol. On one hand, ethanol is then released in the media where it inhibits the growth of prokaryotes and other unicellular organism that cannot assimilate ethanol, creating an advantageous replicative niche for yeast. On the other hand, alcoholic fermentation yields only 2 molecules of ATP for every molecule of glucose metabolized. Thus, alcoholic fermentation is very inefficient compared to oxidative phosphorylation, which produces 36 molecules of ATP for every molecule of glucose. Nonetheless, the proliferative advantage conferred to yeast by alcoholic fermentation has been evolutionally favored over energetic efficiency. The signaling pathway involving PKA ensures that the proper transcriptional program, and thus metabolism, for glucose utilization is turned on in the presence of glucose. Moreover, PKA negatively regulates all other transcriptional programs required for utilization of other carbon sources. In this way, energy saving is maximized. A similar mechanism regulating the utilization of the nitrogen source exists and requires TORC1 signaling.

NITROGEN METABOLISM

When glucose is in excess, the availability and quality of the nitrogen source regulates growth. Yeast cells sense and utilize up to 21 different nitrogenous compounds as nitrogen sources. Among these compounds are amino acids, ammonium, urea, citrulline, ornithine and γ-aminobutyric acid. Yeast exhibits a hierarchical preference for nitrogen sources. The quality of a nitrogen source depends on the transcriptional programs activated/repressed in its presence and on the ability to sustain fast growth rate. The nitrogen sources can be

divided according to their quality in three groups: preferred, non-preferred and intermediate (Godard et al., 2007). In general, preferred nitrogen sources repress the transcriptional programs required for the uptake and catabolism of less-preferred nitrogen sources, and down-regulate autophagy. Preferred nitrogen sources, able to sustain a faster cell growth, are always internalized and consumed before non-preferred nitrogen sources. Preferred nitrogen sources are: alanine ammonium, arginine, asparagine, aspartate, glutamine, glutamate, and serine. Non-preferred nitrogen sources are: isoleucine, leucine, methionine, threonine, tryptophan and tyrosine. The intermediate nitrogen sources are: citrulline, ornithine, phenylalanine, proline, valine, urea and γ-aminobutyric acid.

All of the pathways for the utilization of non-preferred sources of nitrogen feed into a common set of reactions for the production of glutamate and glutamine. The amino and amide groups of glutamate and glutamine are the major nitrogen donors in biosynthetic reactions. From glutamate and glutamine are synthetized all other amino acids and nitrogenous compounds such as nitrogen bases and NAD. The yeast nitrogen metabolism evolves around the availability, production and consumption of glutamate and glutamine, and requires intracellular ammonium.

A set of four reactions constitutes the core of nitrogen metabolism.

1) synthesis of glutamate by combination of ammonium with the citric acid cycle intermediate α-ketoglutarate is catalyzed by the $NADP^+$-dependent glutamate dehydrogenase, encoded by *GDH1* (Grenson et al., 1974).

2) glutamate can then combine with ammonium in a reaction catalyzed by glutamine synthetase (GS), the product of *GLN1* (Mitchell, 1985; Mitchell and Magasanik, 1983). These two reactions constitute the anabolic core of nitrogen metabolism and also constitute the ammonium assimilation route.

3) glutamate synthase (GOGAT), the product of *GLT1*, catalyzes the synthesis of two molecules of glutamate from one molecule each of glutamine and α-ketoglutarate (Cogoni et al., 1995).

4) finally, glutamate is converted to α-ketoglutarate, resulting in ammonium release, by the NAD^+-dependent glutamate dehydrogenase, encoded by *GDH2* (Miller and Magasanik, 1990).

The regulated combination of these reactions allows for glutamate production when glutamine is the sole nitrogen source, and glutamine synthesis when glutamate is the only nitrogen source. When cells are in the presence of abundant ammonium concentrations, reactions (1) and (2) ensure proper synthesis of glutamate and glutamine. Non-preferred sources of nitrogen are converted through these reactions to glutamate and glutamine, which in turn serve as the sources of all cellular nitrogen. The expression of the right set of genes required for nitrogen anabolic and catabolic reactions is subject to complex regulation that involves also TORC1 signaling.

Intriguingly, glutamine is not only a key intermediate in nitrogen metabolism but also an important indicator of the general nutrient status. Glutamine was proposed to constitute the intracellular signal activating TORC1 in response to the nitrogen source (Crespo et al., 2002) (more detailed in section 1.3.3). Inhibition of GS with the specific inhibitor MSX mimicked rapamycin treatment leading to activation of only a subset of targets negatively regulated by TORC1 (NCR and RTG, explained in detail in the next section). Thus, TORC1 and the nitrogen metabolism/regulation are intimately interconnected.

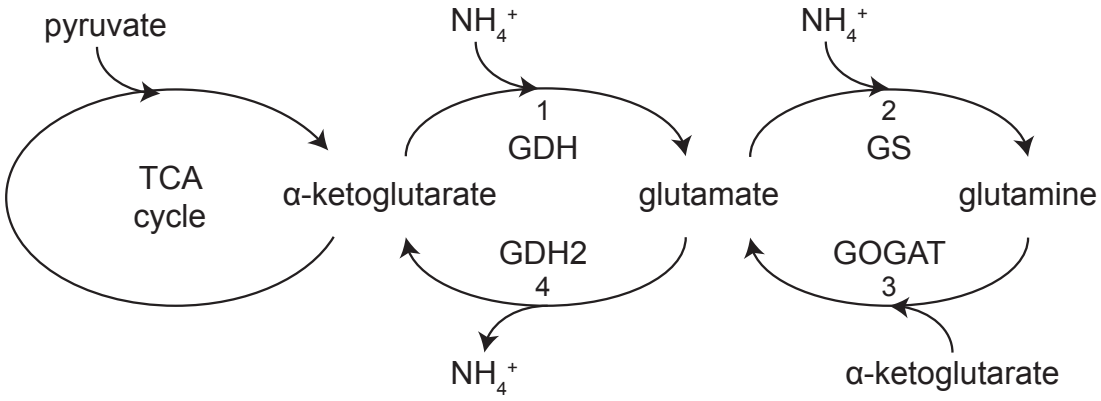

Figure 3: Nitrogen metabolism core reactions

NITROGEN REGULATION

Depending on the availability of the nitrogen source, yeast cells adapt their transcriptional, metabolic, and biosynthetic capabilities to maximize energy gain and growth. Limiting concentrations of nitrogen reduce ribosome biogenesis and translation and result in enlarged G1 phase and slower growth (Brauer et al., 2006). Upon complete removal of nitrogen cells enter a nitrogen-specific quiescent state (Klosinska et al., 2011). The mechanisms involved in the co-ordination of growth rate to the quality and amount of available nitrogen are discussed in the following section.

Permease sorting and nutrient uptake

Nitrogenous compounds enter the cell via permease-mediated transport. The permeases are integral membrane proteins, which are sorted through the secretory system and delivered to the plasma membrane (André, 1995; Regenberg et al., 1999). More than 270 permeases with different specificity are encoded by *S. cerevisiae*. For example, the entry of ammonium into the cell is facilitated by the three permeases Mep1, Mep2, and Mep3 (Marini et al., 1997). A total of 19 amino acid permeases ensure the uptake of amino acids required for protein synthesis or to be used as source of nitrogen (Nelissen et al., 1997). In the presence of good nitrogen source, high affinity permeases are expressed at the plasma membrane. In nitrogen poor conditions, more broad-spectrum general amino acid permeases are expressed. Clear examples of such regulation are the high affinity tryptophan transporter Tat2 and the general amino acid permease Gap1. When cells are shifted from a good to a poor nitrogen source, Tat2 is ubiquitinated, internalized and degraded, while Gap1 is re-directed to the plasma membrane. TORC1 regulates permease sorting via the Tap42-phosphatase branch and possibly by Npr1.

Npr1 is heavily phosphorylated when TORC1 is active and is rapidly dephosphorylated and activated following rapamycin treatment (Gander et al., 2008). Active Npr1 stabilizes several plasma membrane amino acid transporters by antagonizing their ubiquitin-mediated degradation (Beck et al., 1999; Breitkreutz et al., 2010; de Craene et al., 2001; Jacinto et al., 2001; Schmidt et al., 1998; Soetens et al., 2001).

The Tap42-phosphatase pathway is also involved in permease sorting by regulating the stability of the transcription factor Stp1 in response to activation of the SPS-sensing pathway (Shin et al., 2009). The Ssy1-Ptr3-Ssy5 (SPS) sensor is activated by external amino acids presence. Upon activation of SPS, Ssy5 leads to the cleavage and release of two shortened

forms of the transcription factors Spt1 and Spt2. Shortened active Spt1 and Spt2 relocalize to the nucleus where they up-regulate the expression of several amino acid permeases. The other mechanism by which TORC1 regulates permease sorting is via the nitrogen catabolite repression discussed below.

Nitrogen catabolite repression

Preferred nitrogen source repress the transcriptional programs required for the uptake and catabolism of less-preferred nitrogen sources. This phenomenon is known as nitrogen catabolite repression (NCR). The GATA factors are responsible for NCR regulation. This group of DNA binding proteins is composed by the two transcriptional activators Gln3 and Gat1, and by the two transcriptional repressors Dal80 and Gzf3. The cell controls NCR primarily by modulating the nuclear or cytoplasmic localization of the GATA transcriptional activators. This mechanism partially requires Ure2, a cytoplasmic anchoring protein for Gln3. In an *ure2* mutant Gln3 localizes to the nucleus and drives transcription of NCR genes, regardless of the nitrogen source present. On the contrary, Gat1 does not localize in the nucleus in an *ure2* mutant. Therefore, another mechanism different from Ure2 anchoring regulates Gat1 localization. In the presence of a good nitrogen source, both Gln3 and Gat1 are cytoplasmic, and repression of NCR genes is maximal. During nitrogen starvation or in the presence of a poor nitrogen source Gln3 and Gat1 relocalize to the nucleus, bind to the GATA sequences of NCR-responsive genes promoters and drive their transcription.

TOR controls the expression of NCR genes by favoring the formation of the Ure2-Gln3 complex. This involves the Tap42-phosphatase branch signaling of TORC1. Mechanistically, TOR-dependent and independent events lead to Gln3 and possibly Ure2 phosphorylation favoring their association (Beck and Hall, 1999; Cardenas et al., 1999; Carvalho and Zheng, 2003; Hardwick et al., 1999; Tate et al., 2010; 2009).

Retrograde response

The retrograde response is a mitochondrial-to-nucleus signal that drives the transcription of nuclear encoded mitochondrial genes to readjust carbohydrate and nitrogen metabolism. Mitochondria are very important biosynthetic and energetic hubs for the cell. Important metabolic processes such as lipid, nucleotide and amino acid precursor synthesis take place within the mitochondria. Mitochondrial dysfunctions, possibly sensed by a decrease in

the levels of glutamate or glutamine, activate the retrograde response (RTG). The mitochondria are also one of the sites for α-ketoglutarate production, generated from pyruvate and acetyl-CoA by the first three enzymes of the TCA cycle. Transcription of these three enzymes, including *CIT2*, is subject to RTG regulation. Thus, growth on nitrogen sources requiring α-ketoglutarate for assimilation activates RTG (Liu and Butow, 1999).

At the core of the retrograde response pathway are Rtg1 and Rtg3. Rtg1/3 form a heterodimeric transcriptional activator whose nuclear localization is regulated in response to mitochrondrial integrity and nitrogen availability. RTG regulatory pathways impinge on Rtg1/3 localization. Phosphorylated Mks1 binds to Rtg1/3 and sequesters the complex in the cytoplasm. Rtg2 binding to Rtg1/3 allows nuclear translocation and activation of the RTG responsive genes.

TORC1 negatively regulates RTG dependent transcription (Chen and Kaiser, 2003; Komeili et al., 2000; Shamji et al., 2000). Whether TORC1 regulates RTG directly or indirectly is still unclear. TORC1 association with Mks1, and the fact that Mks1 is less phosphorylated following rapamycin treatment, would suggest a direct role for TORC1 in RTG. On the contrary, the observation that RTG is robustly induced in cells with defective mitochondria even in rich medium, and the fact that rapamycin sometimes fails to induce *CIT2* expression depending on the quality of the nitrogen source present in the medium, would speak for an indirect role of TORC1 in RTG control (Tate and Cooper, 2003).

Another nitrogen regulated pathway

Several line of evidence accumulated in the last years suggest that nitrogen-source regulation of NCR and RTG genes does not proceed solely through the TORC1 pathway. First, rapamycin induction of *CIT2* occurs when cells are grown in the presence of ammonium and glutamine, but not in the presence of proline or glutamate (Tate and Cooper, 2003). Second, Gln3 phosphorylation is different in rapamycin-treated versus nitrogen starved or MSX treated cells (Tate et al., 2009). Third, Gln3 and Gat1 nuclear localization occurs in response to different stimuli, despite their overlapping function in regulating NCR. Gln3 activates NCR predominantly following nitrogen limitation or MSX treatment, while Gat1 nuclear localization occurs in response predominantly to rapamycin treatment (Tate et al., 2010). Taken together this data suggest the existence of at least one other nitrogen-signaling pathway, the nature of which has yet to be uncovered.

General amino acid control

Another pathway that controls the transcription of many genes required for multiple amino acid biosynthetic pathways in response to starvation is the general amino acid control (GAAC). Gcn4 is the transcriptional regulator at the core of GAAC. GAAC is efficiently activated in response to increased unloaded tRNAs. Gcn4 is controlled at the translational level by a reinitiation mechanism involving four short upstream open reading frames (uORFs) (Mueller and Hinnebusch, 1986). In normal growth conditions and in the presence of abundant ternary complexes, the four uORFs upstream of *GCN4* ORF are efficiently recognized by the scanning ribosomes and initiate translation. Upon amino acid starvation, multiple tRNAs become deacylated (Zaborske et al., 2009; 2010). Deacylated tRNAs bind to Gcn2 and activate the kinase (Dong et al., 2000; Wek et al., 1989). Active Gcn2 phosphorylates eIF2α, leading to reduced levels of ternary complex. In turn, the scanning efficiency of ribosome to reinitiate translation is reduced. In this way, an increasing proportion of ribosomes reinitiate translation at GCN4 bypassing the four uORFs. Gcn4 is then translated and it activates GAAC responsive genes transcription, to promote amino acids biosynthesis to restore an efficient translation.

NCR and GAAC also appear interlinked, since expression of GCN4 is subject to nitrogen catabolite repression (Godard et al., 2007). Thus, the general amino acid control system is itself a major effector of the TOR pathway, with Gcn4 and Gln3 each inducing a similar number of genes during rapamycin treatment (Staschke et al., 2010).

1.3.3 TORC1 upstream signaling

mTORC1 upstream regulation

The discovery of mammalian TOR (mTOR) and its activation by nutrients (amino acids) and growth factors (insulin) strengthened the role of TOR as central controller of cell growth in eukaryotes (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). Two signaling pathways cooperatively converge on mTORC1 activation: the growth factor pathway and the amino acid sensing pathway.

Growth factor stimulation of mTOR requires the PI3K/Akt signaling branch. Upon growth factor stimulation, such as insulin, the activity of phosphatidylinositol 3-kinase is increased, leading to the production of phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 synthesis is antagonized by the lipid phosphatase PTEN. Increasing levels of PIP_3 activate PDK1 (3-phosphoinositide dependent protein kinase-1), which phosphorylates the activation loop of Akt (Thr308). Active Akt phosphorylates TSC2, inhibiting the tuberous sclerosis complex (TSC), which acts as GAP for the small GTP binding protein Rheb. Thus, growth factors stimulation leads to the GTP loading and activation of Rheb. Rheb was shown to localize to endomembranes (Buerger et al., 2006; Saito et al., 2005; Sancak et al., 2008; Takahashi et al., 2005). Lysosomal active Rheb could bind to the N-terminal portion of the kinase domain of mTOR increasing mTOR activity by enhancing substrates recruitment (Long et al., 2005b; 2005a; Sato et al., 2009). Interestingly, loss of PTEN and activating mutations in PI3K are one of the most frequent genetic mutations in human cancer, underlying the importance of tight control over mTOR activation.

Amino acids dictate mTORC1 lysosomal localization. In the absence of aminoacids, mTORC1 is cytoplasmic and does not interact with Rheb (Long et al., 2005b). Upon restimulation with amino acids mTORC1 punctae appear on the lysosomal membrane and mTOR interacts and is activated by Rheb. Thus, amino acids sufficiency plays an important role in mTOR activation by growth factors. It is also noteworthy that the amino acids sensing pathway per se (in the absence of active Rheb) is unable to activate mTOR (Sancak et al., 2008). Thus only the simultaneous presences of growth factors, leading to Rheb activation, and amino acids, which tether mTOR to the Rheb-containing membranes, achieve mTOR activation. The mechanism regulating mTORC1 tethering on the lysosomal surface was lately uncovered and is the subject of intensive research.

At the center of the amino acid sensing pathway are the Rag proteins (Sancak et al., 2008; 2010). The Rags are Ras-related small GTP binding proteins. Four Rag proteins exist: RagA, B, C and D. RagA with RagB, and

RagC with RagD constitute two pair of homologues. Strikingly, Rag proteins activation requires the formation of Rag etherodimers. Always one of the RagA/B pair was found in complex with one of the RagC/D pair. To add more complexity to Rags regulation, only one guanosine nucleotide binding combination lead to the active complex conformation. This happens when RagA/B binds GTP and RagC/D binds GDP. The complex results inactive in the RagA/B-GDP and RagC/D-GTP conformation. Rag complexes simultaneously loaded with two GTPs or two GDPs were never observed. Only in the active conformation Rags tether mTORC1 to the lysosomal membrane. Amino acid sufficiency leads to formation of the active Rag complex. Different mechanisms explaining Rag activation and hence mTORC1 activation by amino acids were proposed.

The first mechanism described is the so-called “V-ATPase dependent inside-out amino acid sensing mechanism” (Zoncu et al., 2011). In early studies, it was shown that the pentameric Ragulator complex formed by p18, p14, MP1, HBXIP and C7orf59 (now renamed LAMTOR1-5) was required for Rag activation. Ragulator activated RagA/B acting as GEF (Bar-Peled et al., 2012). Ragulator GEF activity did not reside in one single component, but rather in the whole complex. The mechanism leading to Ragulator activation required the vacuolar ATPase and luminal amino acid accumulation, but was independent of the proton gradient generated.

The second mechanism proposed for Rags activation involves glutaminolysis (Durán et al., 2012). Glutaminolysis converts in a two-step reaction glutamine to α-ketoglutarate. In the first step, catalyzed by the glutaminase (GLS), intracellular glutamine is converted to glutamate. In the second step glutamate dehydrogenase (GDH) converts glutamate to α-ketoglutarate. Increasing α-ketoglutarate levels led to GTP loading of RagA/B and thus, mTORC1 activation in response to amino acids. The important feature of this model is that it combines glutamine and leucine sensing, the two most potent amino acid inducers of mTORC1 activity. The importance of leucine in this model is highlighted by the requirement of leucine as cofactor for GDH activity (Carobbio et al., 2009; Li et al., 2003).

In the last year an octomeric complex interacting with Rags at the lysosomal surface was identified. This complex was shown to posses GAP activity towards RagA/B and has therefore renamed GATOR (GTPase-activating protein activity toward RAGs) (Bar-Peled et al., 2013). GATOR was additionally dissected in two sub-complexes. GATOR1 components depletion promoted constitutive localization of mTORC1 to the lysosomal membrane and blocked mTORC1 inactivation following amino acid withdrawal, suggesting that GATOR1 inhibits mTORC1 signaling. On the other hand depletion of GATOR2 components prevented mTORC1 translocation to the lysosome and impaired amino acid–induced activation of mTORC1. Taken together these

data indicate that the GATOR sub-complexes reciprocally regulate mTORC1-dependent amino acid sensing. More specifically GATOR1 functioned downstream of GATOR2 and upstream of Rags in the amino acid sensing of mTORC1. Intriguingly, inactivating mutations and truncating deletions in the genes encoding the GATOR1 proteins DEPDC5 and nitrogen permease regulator-like 2 (NPRL2) were detected in human glioblastoma and ovarian tumors.

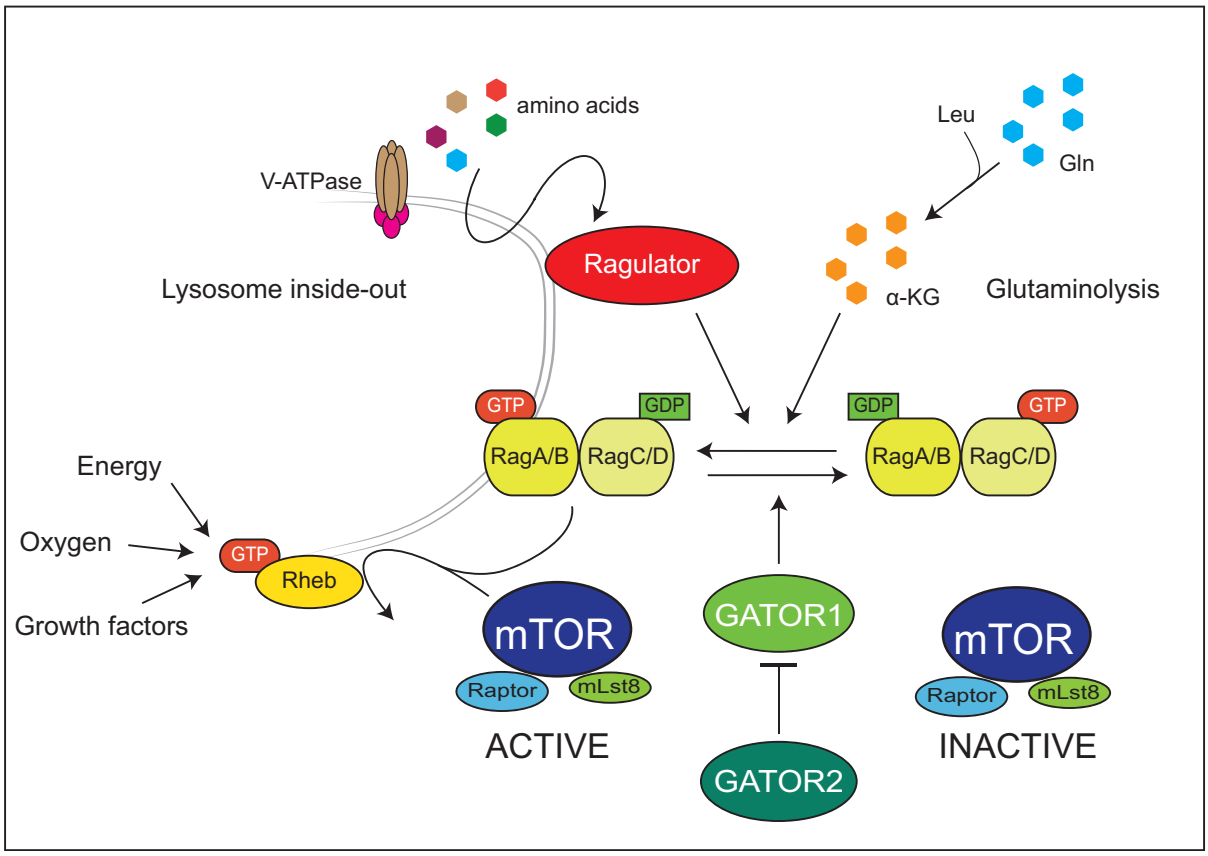

Figure 4: mTORC1 upstream signaling

Yeast TORC1 upstream regulation

Yeasts are insensitive to growth factor stimulation. *S. cerevisiae* does not encode TSC homologs, and Rheb is not involved in TORC1 signaling. The more ancient nutrient sensing activation of TORC1 is conserved and partially overlaps with mTOR activation by amino acids. Thus, TOR couples nutrient availability (carbon and mostly nitrogen source) to cell growth. But which is the signal impinging on TORC1? And how does the nitrogen source influence TORC1 activity?

Yeast TORC1 activation by the nitrogen source is still elusive, but similarities between yeast and mammalian amino acid sensing arose in the last few years. In a first study, glutamine, the preferred nitrogen source of yeast cells, was proposed to constitute the intracellular signal transmitting nitrogen sufficiency to TORC1 (Crespo et al., 2002). Glutamine in yeast is synthetized by the essential gene *GLN1*, encoding for the cytosolic glutamine synthetase (GS). Inhibition of glutamine synthesis with the specific GS inhibitor MSX (methionine sulfoximine) led to decrease intracellular glutamine levels and arrest in cell cycle equivalent to rapamycin treatment. Some readouts of TORC1, the transcription factors Gln3, Rtg1 and Rtg3 were affected by MSX treatment, while other TORC1 readouts such as Gat1, Msn2/4 were unaffected. In mammalian cells, TOR responds to essential amino acids used for protein synthesis (Hara et al., 1998; Wang et al., 1998). In yeast, TOR may respond to glutamine both as an amino acid required for protein synthesis and as a nitrogen source. The finding that only a portion of TORC1 readouts is affected by intracellular glutamine levels confirms the hypothesis that TORC1 acts as a multichannel processor to differentially regulate gene expression in response to different nutrient conditions (Shamji et al., 2000).

In a more recent paper, the yeast homolog of the Rag proteins, Gtr1 and Gtr2 were shown to be important for TORC1 activation in response to amino acid availability (Binda et al., 2009). Gtr1 and Gtr2 were found in complex with two other proteins, Ego1 and Ego3. *EGO1*, *EGO3* and *GTR2* deletions were all identified in a selection for mutants that are unable to exit from a rapamycin-induced growth arrest (Dubouloz et al., 2005). All together these proteins form the tetrameric EGO complex. Ego1 and Ego3 act as functional homolog of the Ragulator complex and are involved in complex stability (Kogan et al., 2010). The EGO complex (EGOC) was found on the vacuolar membrane, the same sub-cellular localization as TORC1. Active EGOC, analogously to Rags, required Gtr1-GTP and Gtr2-GDP binding. EGOC signaling to TORC1 was so far studied the most in response to leucine starvation and restimulation. Leucine starvation destabilized the Gtr1-TOR interaction leading to decreased phosphorylation of Sch9, a direct target of

TORC1. Expression of a GTP locked version of Gtr1 ($Gtr1^{Q65L}$) led to delayed Sch9 dephosphorylation during leucine starvation. Cells expressing the dominant negative version of Gtr1 locked in a GDP binding conformation ($Gtr1^{S20L}$) were reported to be sick. In another study, leucyl-tRNA synthetase (LeuRS) was proposed to regulate TORC1 activity via the EGOC (Bonfils et al., 2012). The molecular mechanism involved the interaction between the editing site of LeuRS and Gtr1. In the presence of intracellular leucine, the interaction LeuRS-Gtr1 protected Gtr1-GTP loading, inhibiting GTP hydrolysis. The GEF and GAP for Gtr1 were identified as Vam6 and the Npr2/Npr3/Iml1 containing complex (Binda et al., 2009; Panchaud et al., 2013).

Vam6 is a vacuolar protein that plays a critical role in the tethering steps of vacuolar membrane fusion by facilitating guanine nucleotide exchange on small guanosine triphosphatase Ypt7 (Oka et al., 2004; Raymond et al., 1992; Robinson et al., 1988; Rothman et al., 1989; Wurmser et al., 2000). It was later demonstrated that Vam6 also functioned as GEF for Gtr1 in vitro and in vivo (Binda et al., 2009). Vam6 loss decreased Gtr1-TORC1 interaction. Moreover, unaltered TORC1 signaling was observed in a *ypt7* strain, which displays the same abnormal vacuolar fragmentation as a *vam6* strain. Thus, in addition to its regulatory role in homotypic vacuolar fusion and vacuole protein sorting as part of the HOPS complex, Vam6 also controls the activity of TORC1 by activating the Gtr1 subunit of the EGOC.

Early evidence suggested that Npr2 and Npr3 positively regulate non-nitrogen-starvation (NNS)-induced autophagy by negatively regulating TORC1 (Neklesa and Davis, 2009; Wu and Tu, 2011). NNS-autophagy is a particular form of autophagy triggered when cells are switched from a rich medium to a minimal medium in the complete absence of nitrogen starvation. Further more, Npr2 and Npr3 were found in association with other proteins in the large SEA complex. The SEA is a coatomer-related complex that associates dynamically with the vacuole (Dokudovskaya et al., 2011). In a later study the SEA complex was genetically dissected. It emerged that it could be divided in two sub-complexes possessing different effects on TORC1 activation. The first complex, containing Npr2, Npr3 and Iml1 acted as GAP for Gtr1, thus downregulating TORC1 activity. The complex was therefore named SEACIT (for SEAC subcomplex inhibiting TORC1 signaling) and is the homolog of GATOR1 in mammalian cells. The second complex, formed by Sea2, Sea3, Sea4, Seh1, and Sec13, antagonized the negative regulation of SEACIT. Renamed SEACAT (for SEAC subcomplex activating TORC1 signaling) it constitutes the homolog of GATOR2 in higher eukaryotes (Panchaud et al., 2013).

Overall these data highlight the parallelism existing between Rags and EGOC in the signaling of amino acids sufficiency to (m)TORC1. But few considerations on the differences existing between the two upstream signaling

pathway should be taken into account. First, TORC1 activation by the EGOC was dependent on the presence of Tco89, a nonessential component of TORC1. If the EGOC were essential for TORC1 activity, one would expect it being dependent on essential components of the pathway. Second, Gtr1 deletion is not lethal and induces slow growth and decreased Sch9 phosphorylation. Third, in $Gtr1^{Q65L}$ expressing cells TORC1 activity was still repressed by ammonium starvation. Fourth, the SEACIT inhibition of TORC1 likely does not apply during nitrogen starvation, since Npr2 and Npr3 are clearly involved in NNS-induced autophagy. Fifth, the majority of the experiments supporting EGO complex signaling to TORC1 were always performed in the presence/absence of leucine. The importance of EGOC signaling in the presence/absence of other amino acids was never thoroughly examined (e.g. glutamine, the other proposed upstream signal of TORC1). Therefore, it appears that EGOC is sufficient to explain, at least partially, amino acids sensing signaling to TORC1, but is not sufficient to explain the nitrogen sensing signaling to TORC1. It is tempting to speculate that amino acid and nitrogen source sensing are separate and act independently on TORC1. The analysis of this point will be the focus of the results section.

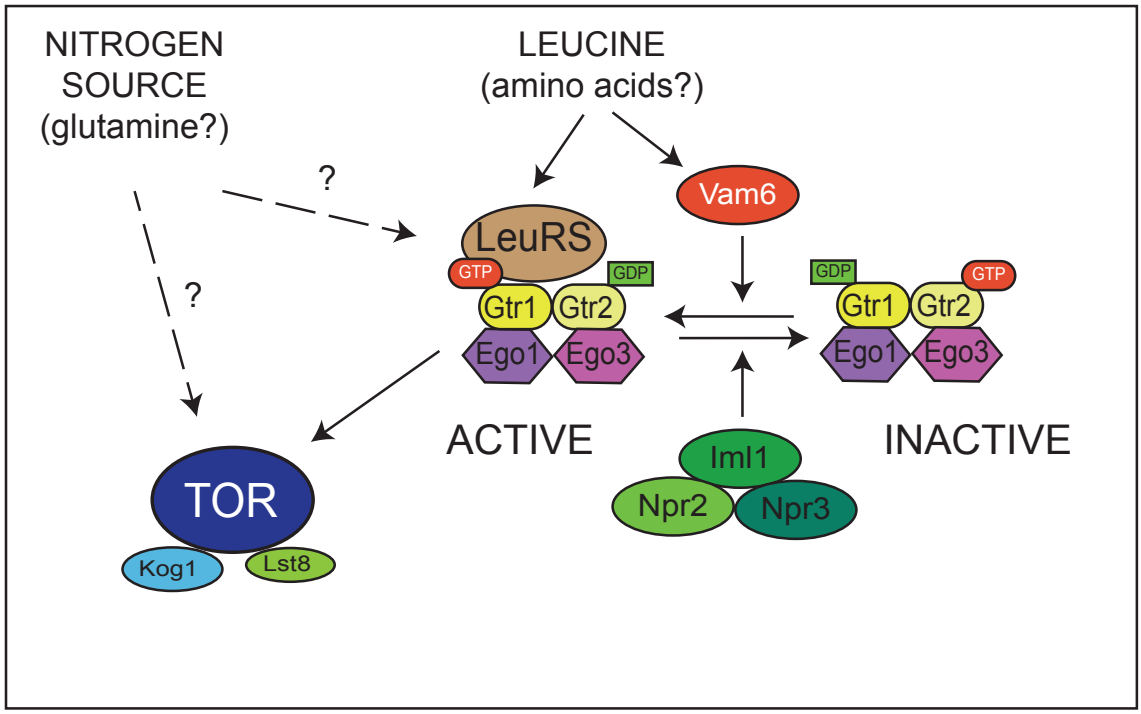

Figure 5: TORC1 upstream signaling

1.4 AIM OF THE STUDY

As outlined in the introduction, the upstream regulation of TORC1 in response to the nitrogen source is only partially understood. The signaling deriving from amino acid sufficiency is only partly transmitted to TORC1 by the EGO complex. A unifying model of how the nitrogen source (amino acids and other nitrogenous compounds such as ammonium) is sensed and activates TORC1 is still missing.
The two major aims of the study are: 1) clarifying possible differences in the sensing of nitrogen source versus amino acid sufficiency; 2) determining the signaling route leading to TORC1 activation in response to the nitrogen source.

2 RESULTS

The following manuscript published on The Journal of Biological Chemistry (JBC) forms the basis of my PhD thesis. The cumulative bibliography from the accepted paper and the thesis is given on page 81.

Nitrogen Source Activates TOR (Target Of Rapamycin) Complex 1 Via Glutamine And Independently Of The Gtr/Rag Proteins

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2.1 CAPSULE

Background: Nutrients, in particular the nitrogen source, activate TORC1 signaling in yeast.
Results: The nitrogen source stimulates a rapid, transient activation of TORC1. Preferred nitrogen sources result in sustained TORC1 activity and growth, via a mechanism dependent on glutamine accumulation and independent of the Gtr/Rag.
Conclusions: Nutrients activate yeast TORC1 via two distinct mechanisms.
Significance: Gtr/Rag is not the only mechanism to activate TORC1 in response to nutrients.

2.2 ABSTRACT

The evolutionary conserved TOR complex 1 (TORC1) activates cell growth in response to nutrients. In yeast, TORC1 responds to the nitrogen source via a poorly understood mechanism. Leucine, and perhaps other amino acids, activates TORC1 via the small GTPases Gtr1 and Gtr2, orthologs of the mammalian Rag GTPases. Here we investigate the activation of TORC1 by the nitrogen source and how this might be related to TORC1 activation by Gtr/Rag. The quality of the nitrogen source, as defined by its ability to promote growth and glutamine accumulation, directly correlates with its ability to activate TORC1 as measured by Sch9 phosphorylation. Preferred nitrogen sources stimulate rapid, sustained Sch9 phosphorylation and glutamine accumulation. Inhibition of glutamine synthesis reduces TORC1 activity and growth. Poor nitrogen sources stimulate rapid but transient Sch9 phosphorylation. A Gtr1 deficiency prevents the transient stimulation of TORC1 but does not affect the sustained TORC1 activity in response to good nitrogen sources. These findings suggest that the nitrogen source must be converted to glutamine, the preferred nitrogen source in yeast, to sustain TORC1 activity. Furthermore, sustained TORC1 activity is independent of Gtr/Rag. Thus, the nitrogen source and Gtr/Rag activate TORC1 via different mechanisms.

2.3 INTRODUCTION

To avoid metabolic stress, unicellular organisms possess finely tuned regulatory systems to ensure that cell growth and replication are tightly coupled to nutrient availability (Smets et al., 2010). Nitrogen is an essential element required for synthesis of amino acids, nucleotides and other cellular components. The budding yeast *Saccharomyces cerevisiae* can sense, take up and assimilate several different nitrogen sources (N-sources) (Magasanik and Kaiser, 2002). Qualitatively better N-sources are assimilated before others. The quality of a N-source is generally defined by its ability i) to sustain core nitrogen metabolism and thus growth and ii) to activate or repress specific transcriptional programs that mediate selective nitrogen assimilation (Godard et al., 2007). To maintain growth, N-sources must sustain core nitrogen metabolism that involves four interconnected enzymatic reactions with α-ketoglutarate, glutamate, glutamine and ammonia as key metabolites (Cooper, 1982). Glutamine is considered the best N-source for yeast cells (Cooper, 1982). In the presence of exogenous glutamine as the N-source, yeast cells grow and replicate at a high rate, and maximally repress the metabolic pathways required for utilization of other N-sources (Hofman-Bang, 1999; Magasanik and Kaiser, 2002). Moreover, glutamine plays an essential role in anabolic metabolism, in particular in purine and pyrimidine synthesis, and in the biosynthesis of other amino acids following conversion to glutamate. Amino acids other than glutamate and glutamine can also serve as nitrogen sources, but only by sustaining nitrogen core metabolism indirectly. These amino acids undergo deamination, liberating free ammonia which can then be used for glutamate or glutamine synthesis, or generate glutamate via transamination (Magasanik, 1992). Depending on the amino acid, the remaining carbon skeleton following deamination or transamination is either fed into carbon metabolism or converted through the Ehrlich pathway into fuse oils and secreted (Hazelwood et al., 2008).

Metabolic adaptation to the nutritional environment is achieved via complex transcriptional, translational and post-translational regulation which relies on nutrient sensing and signal transduction (De Virgilio and Loewith, 2006; Ljungdahl and Daignan-Fornier, 2012). The target of rapamycin (TOR) pathway is a central regulator of cell growth in response to nutrients (Loewith and Hall, 2011; Wullschleger et al., 2006). TOR is an evolutionary conserved protein kinase found in two highly conserved multi-protein complexes termed TOR complex 1 (TORC1) and TORC2. TORC1 is acutely sensitive to rapamycin and is activated by nutrients (Barbet et al., 1996; Beck and Hall, 1999; Smets et al., 2010). TORC1 couples nutrient sufficiency to growth by

activating anabolic processes such as protein synthesis and ribosome biogenesis, and repressing catabolic processes such as autophagy. Despite the many cellular functions in which TORC1 is involved, few direct TORC1 targets are known. The best-characterized TORC1 direct target in yeast is Sch9, a kinase belonging to the AGC kinase family also containing PKA, PKG and PKC. In the presence of nutrients, TORC1 phosphorylates at least six residues in the Sch9 C-terminus (C-term) and thereby activates Sch9 in a rapamycin sensitive manner (Huber et al., 2009; Magasanik and Kaiser, 2002; Soulard et al., 2010; Urban et al., 2007). Sch9 activates genes encoding ribosomal proteins and factors required for ribosome biogenesis (RiBi regulon) (Godard et al., 2007; Huber et al., 2011), ultimately controlling cell growth and longevity (Cooper, 1982; Fabrizio et al., 2001; Kaeberlein et al., 2005; Toda et al., 1988).

In yeast, the so-called EGO complex (EGOC) mediates amino-acid-dependent activation of TORC1 (Binda et al., 2010; Cooper, 1982). EGOC consists of Gtr1, Gtr2, Ego1 and Ego3. The small GTP binding proteins Gtr1 and Gtr2 are orthologs of the mammalian RagA/B and RagC/D GTPases, respectively (Binda et al., 2009; Hofman-Bang, 1999; Kim et al., 2008; Magasanik and Kaiser, 2002; Sancak et al., 2008). Like their mammalian counterparts, Gtr1 and Gtr2 form a heterodimer that is active in the $Gtr1^{GTP}$-$Gtr2^{GDP}$ conformation. Ego1 and Ego3, the functional homologs of the mammalian Ragulator complex (Laplante and Sabatini, 2012; Magasanik, 1992), are also required for activation of Gtr. The amino acid leucine stimulates $Gtr1^{GTP}$ interaction with Ego1, thereby activating EGOC and, in turn, TORC1 (Binda et al., 2009; Hazelwood et al., 2008). Vam6 and the Npr2/Npr3/Iml1 complex were identified as the guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP), respectively, for Gtr1 (Binda et al., 2009; De Virgilio and Loewith, 2006; Ljungdahl and Daignan-Fornier, 2012; Panchaud et al., 2013). The yeast leucyl-tRNA synthetase (LeuRS) was reported to positively influence the GTP loading of Gtr1 (Bonfils et al., 2012; Loewith and Hall, 2011; Wullschleger et al., 2006).

The rapamycin induced response in yeast mimics the response to nitrogen starvation (Cardenas et al., 1999; Hardwick et al., 1999; Shamji et al., 2000). This early observation led to the conclusion that the N-source activates TORC1. The mechanism by which the N-source, in the form of an amino acid or other nitrogenous compound, activates TORC1 is poorly understood. Early studies reported a prominent role for the intracellular level of glutamine in TORC1 activation, at least toward the transcription factors Gln3, Rtg1 and Rtg3 (Crespo et al., 2002). Although increasing evidence suggests that intracellular leucine stimulates TORC1 via EGOC, it is not yet known if the N-source, including ammonium or other amino acids, activates TORC1 via EGOC. Cells expressing a constitutively active version of Gtr1 ($Gtr1^{Q65L}$) still

down-regulate TORC1 activity in response to ammonium starvation (Binda et al., 2009), suggesting that the N-source signals to TORC1 independently of Gtr1.

Here we investigate the modulation of TORC1 activity by different N-sources in vivo, using the phosphorylation state of Sch9 as readout. We show that preferred N-sources sustain TORC1 activity and growth rate by rapidly increasing glutamine synthesis. Moreover, we demonstrate that glutamine synthesis does not require Vam6 or Gtr1 for TORC1 activation. Thus, there are distinct molecular mechanisms by which nutrients activate TORC1.

2.4 RESULTS

TORC1 rapidly phosphorylates Sch9 in response to the nitrogen source. We initially sought to establish a system, using the phosphorylation state of the Sch9 C-term as a readout, to monitor changes in TORC1 activity in response to nutrients in vivo. Moreover, we reasoned that signaling events associated with nutrient availability would be triggered very rapidly, as in the case of glucose-induced cAMP production in yeast cells (Thevelein and De Winde, 1999; van der Plaat, 1974). To capture fast acting signaling events, Sch9 phosphorylation was assayed as early as 20 seconds, and up to 4 hours, after stimulation with nutrients. We used the strain YSBN9 (Canelas et al., 2010) which we made prototrophic by transformation with the centromeric plasmid p*SCH9*-3HA containing the selectable marker *URA3* in addition to expressing C-terminally 3xHA tagged Sch9 under the control of its own promoter. By working with a prototrophic strain, we were able to grow cells in medium supplemented with a single nitrogen source, thereby avoiding the confounding effect of the simultaneous presence of different N-sources.

We first validated our system by pharmacologically inhibiting TORC1. Rapamycin treatment of yeast cells, grown in the presence of glutamine, resulted in an acute decrease in Sch9 C-term phosphorylation. Compared to untreated cells, Sch9 phosphorylation decreased to minimal levels within 5 minutes and remained low for the remainder of the experiment (Fig. 6A and B). We next sought to determine the effect of changes in the nutritional environment on TORC1 activity by varying the quality of nutrients from good to poor (nutrient downshift) or from poor to good (nutrient upshift). When performing nutrient downshift experiments, cells were grown in the presence of the preferred N-source glutamine and switched to medium containing a less preferred N-source such as proline. As observed for rapamycin treatment, Sch9 phosphorylation decreased to minimal levels within minutes after nutrient downshift (Fig. 6C and D). These data underscore the similarity between

pharmacological and physiological inhibition of TORC1. In the case of nutrient upshift experiments, cells were grown in the presence of proline and stimulated with a good N-source. When using glutamine as the good N-source, Sch9 phosphorylation levels increased more than two fold already within one minute after addition of glutamine (Fig. 6E and F). Following this initial increase, Sch9 phosphorylation decreased to rise again at 15 minutes. Pre-treating cells with rapamycin for 10 minutes was sufficient to block the increase in Sch9 phosphorylation induced by glutamine (Fig. 6G), demonstrating that Sch9 was phosphorylated by TORC1 during the nutrient upshift. To determine whether the increase in Sch9 phosphorylation was caused exclusively by the presence of the newly introduced N-source, we performed mock upshift experiments without increasing the nitrogen quality. As expected, no increase in Sch9 phosphorylation was observed when cells were treated with water or the same medium in which they were growing (Fig. 6H and I). Overall, these findings indicate that our in vivo system is valid and that the N-source rapidly stimulates TORC1 activity.

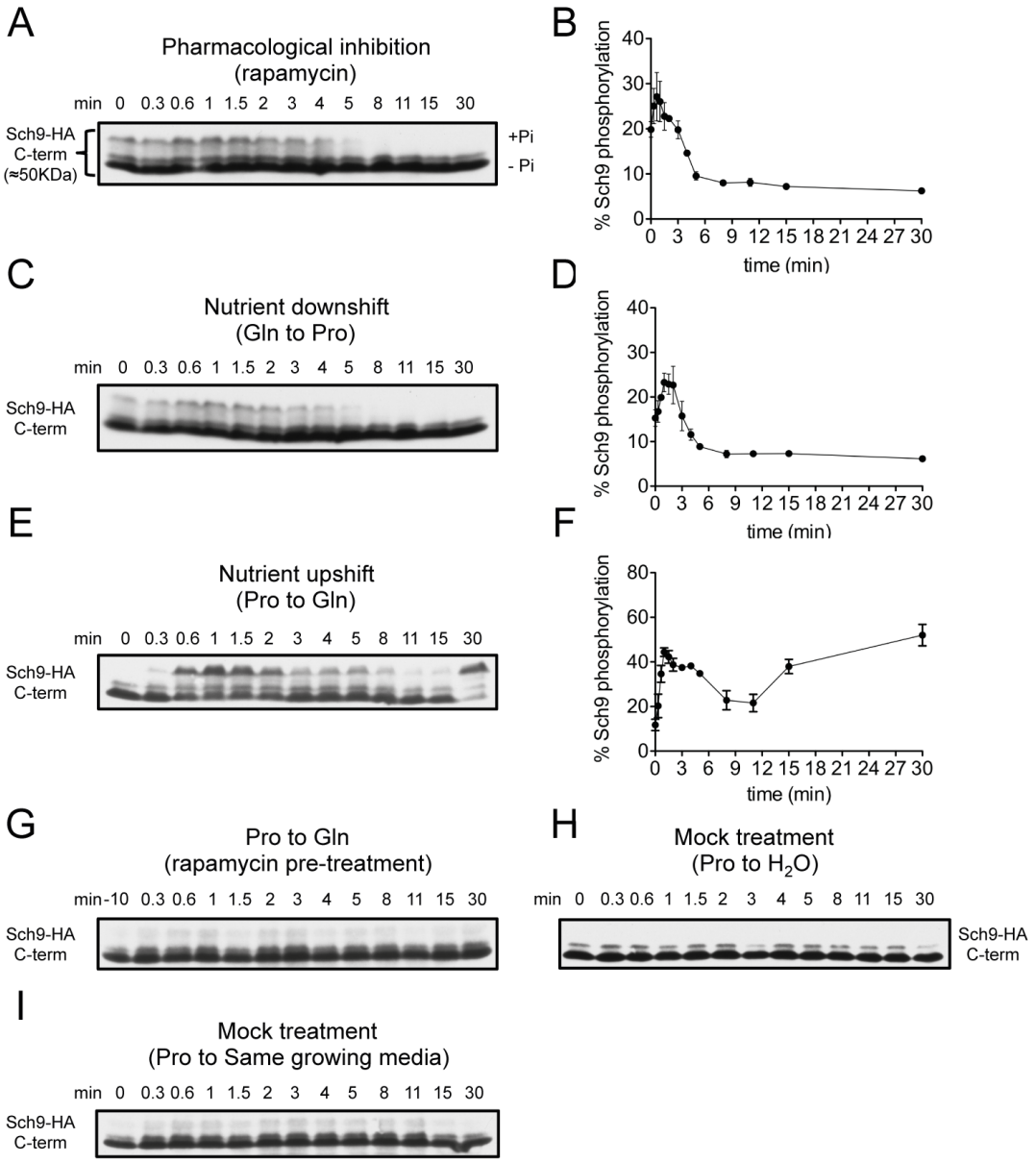

Figure 6: TORC1 rapidly phosphorylates Sch9 in response to the nitrogen source.

(A and B) Sch9 C-term phosphorylation decreases upon rapamycin treatment (200 nM) and (C and D) during nutrient downshift (glutamine to proline). (D and E) Sch9 C-term phosphorylation increases during nutrient upshift (proline to glutamine). Sch9 phosphorylation is unaffected when cells are subjected to either (G) nutrient upshift (Pro to Gln) following rapamycin pre-treatment (200 nM, 10 min), or mock treated with (H) water or (I) mock treated with the same medium in which they were growing.

Protein samples were chemically cleaved with NTCB. Electrophoretic mobility shift induced by Sch9 C-term phosphorylation state assessed by Western blot (panel A, C, E, G, H and I). Relative quantification of Sch9 C-term phosphorylation (panel B, D and F) is expressed as mean ± SEM of at least 3 independent biological experiments.

The quality of the nitrogen source determines TORC1 activity. To gain mechanistic insight into the nutrient-induced activation of TORC1, we quantified the extent of TORC1 activity over time in response to different N-sources. We determined the dynamics of Sch9 phosphorylation during nutrient upshift experiments in response to various nitrogen sources including growth-promoting amino acids and ammonium (Fig. 7). All N-sources tested induced a rapid increase in Sch9 phosphorylation within 20 seconds and peaking at 2-3 minutes after stimulation, although with different amplitudes. As observed above for glutamine, this rapid increase was transient as Sch9 phosphorylation then decreased, often to near basal levels. Upon comparing the change in Sch9 phosphorylation at 30 minutes and up to 4 hours after shift, the N-sources clustered into two discrete groups. The first group contained the so-called high-end N-sources, able to re-stimulate and sustain high TORC1 activity ($p < 0.05$). The second group contained the low-end N-sources where Sch9 phosphorylation remained low (Fig. 7A). Arginine, asparagine, glutamine, and ammonium belonged to the high-end N-sources (Fig. 7B). Serine, threonine, the branched chain amino acids (isoleucine, leucine and valine), the negatively charged amino acids (aspartate and glutamate), and the hydrophobic amino acids (alanine, methionine, phenylalanine and tryptophan) fell in the low-end group (Fig. 7C). Tyrosine was not included in the study because of its low solubility in water. All the N-sources belonging to the high-end group were previously characterized as preferred N-sources (Godard et al., 2007). Similarly, the N-sources belonging to the low-end group were previously classified as less preferred, with the exception of alanine, serine, aspartate and glutamate. We conclude that sustained TORC1 activity after nutrient upshift correlates with the quality of the N-source.

We next asked whether the quality of the initial N-source would affect the extent of TORC1 stimulation upon nutrient upshift. To this end, we compared the ability of glutamine and ammonium, two preferred N-sources, to stimulate TORC1 activity in cells initially grown in the presence of proline or leucine, an intermediate and a non-preferred N-source, respectively. We observed that the glutamine- or ammonium-induced increase in Sch9 phosphorylation was equal in cells originally grown in the presence of proline or leucine as the only N-source (data not shown). Therefore, TORC1 activity is solely dependent on the quality of the N-source added during nutrient upshift. Collectively, we conclude that the extent of TORC1 stimulation in vivo parallels the quality of the N-source. In general, preferred N-sources stimulate and sustain TORC1 activity whereas poor N-sources do not.

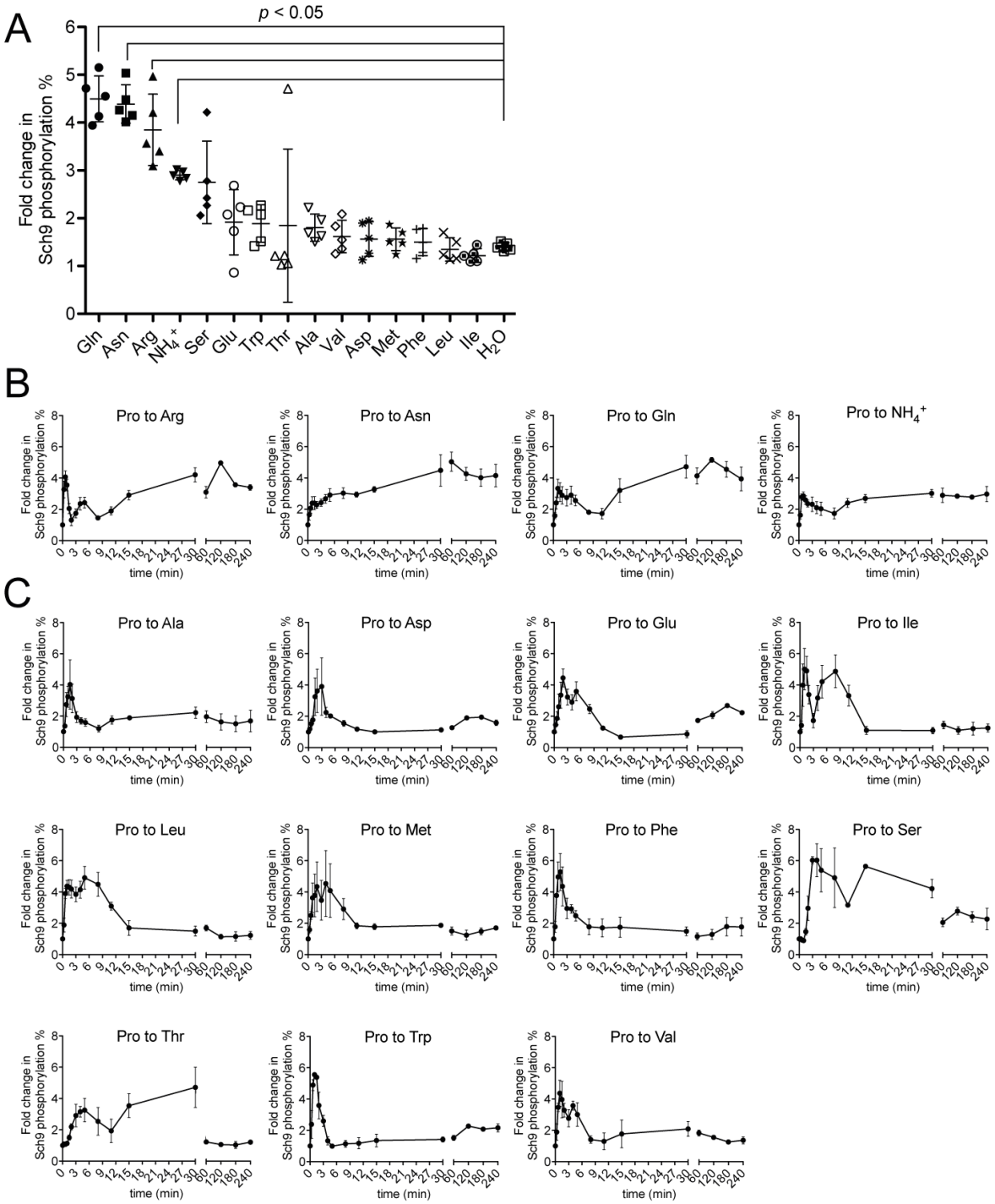

Figure 7: The quality of the nitrogen source determines TORC1 activity.
N-sources can be grouped based on the increase in Sch9 C-term phosphorylation upon nutrient upshift. (A). Classification of the N-sources based on their ability to sustain TORC1 activity for up to 4 hours after shift. The fold change mean value ±SD of Sch9 phosphorylation at five different time points (30, 60, 120, 180 and 240 minutes after shift) for each N-source is shown in the graph. For statistical analysis, we compared in a two-way ANOVA analysis the values of this five time points for each N-source to the respective values obtained in mock shift experiments with water. In four cases (Arg, Asn, Gln and NH_4^+) the difference was significant ($p < 0.05$). (B) Fold change of Sch9 C-term relative phosphorylation following nutrient upshift from

proline to individual high-end N-sources (arginine, asparagine, glutamine, and ammonium). Relative quantifications are expressed as mean ± SEM of at least 3 independent biological experiments. (C) Fold change of Sch9 C-term relative phosphorylation following nutrient upshift from proline to individual low-end N-sources (alanine, aspartate, glutamate, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan and valine). Relative quantifications are expressed as mean ± SEM of at least three independent biological experiments.

The quality of the nitrogen source couples TORC1 activation to cell growth. Phosphorylation of Sch9 influences its kinase activity in vitro and in vivo (Huber et al., 2011; Urban et al., 2007). We next asked whether the Sch9 phosphorylation increase observed during nutrient upshift would result in increased Sch9 kinase activity. To monitor Sch9 kinase activity in vivo, we measured the phosphorylation state of Dot6, a direct target of Sch9 involved in ribosome biogenesis (Huber et al., 2011). As assayed by immunoblotting, nutrient upshifts with glutamine, leucine or ammonium resulted in the appearance of a slow-migrating form of C-terminally 3xHA tagged Dot6 (Fig. 8A). As expected, no detectable change in Dot6 phosphorylation was observed when supplementing cells with water (Fig. 8A). In all three nutrient upshift experiments, Dot6 phosphorylation increased already at 2-3 minutes following stimulation, consistent with the rapid increase in Sch9 phosphorylation described above. Interestingly, Dot6 phosphorylation was sustained up to 30 minutes after stimulation with the preferred N-source glutamine or ammonium, whereas Dot6 phosphorylation decreased starting at 8 minutes after shift to the less preferred N-source leucine. For unknown reasons, shift to leucine also caused a decrease in Dot6 protein levels at 30 minutes after the shift. These data suggest that nutrient stimulation rapidly activates Sch9 downstream of TORC1.

Active Sch9 positively regulates ribosome biogenesis thereby increasing cell growth potential (Jorgensen et al., 2002; Urban et al., 2007). We tested the physiological impact of TORC1 activation upon nutrient upshift on cell growth. Yeast cells grown in the presence of proline were supplemented with glutamine, ammonium, leucine or water (Fig. 8B). Following glutamine or ammonium stimulation, growth increased within 2-3 hours after treatment compared to un-stimulated cells. The observed increase in growth rates paralleled sustained Sch9 and Dot6 phosphorylation up to 4 hours after treatment (Fig. 8C and D). Conversely, growth rate was unaffected when cells were supplemented with leucine or water, and Sch9 and Dot6 phosphorylation remained low. Thus, stimulation with preferred N-sources confers an increase in TORC1 activity and a corresponding increase in proliferation.

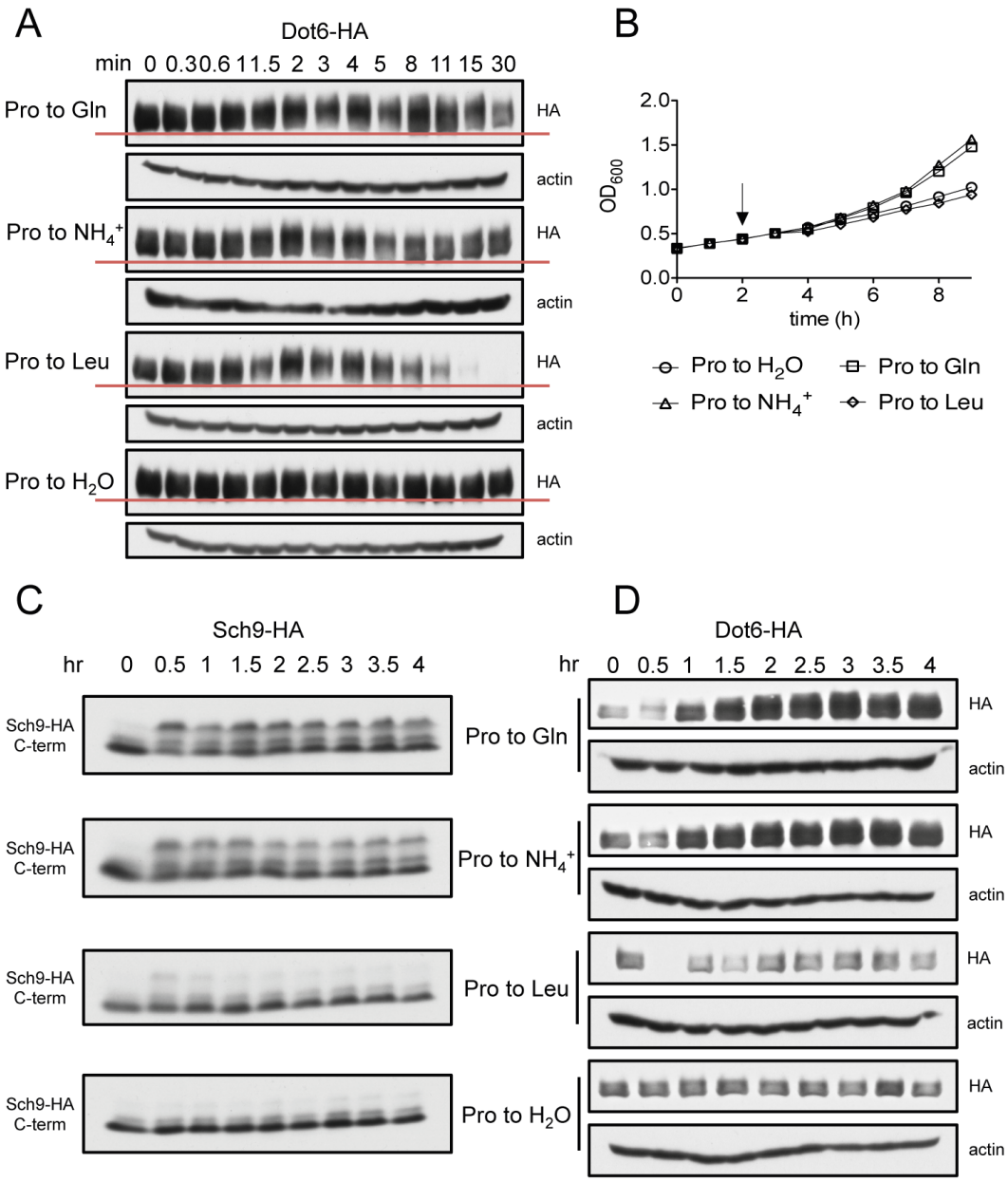

Figure 8: The quality of the nitrogen source couples TORC1 activation to cell growth.
Sch9 is activated following nutrient upshift. (A) The phosphorylation of the Sch9 direct target Dot6 increases during nutrient upshift from proline to glutamine, leucine, ammonium, but not when treating cells with water. A representative Western blot of protein extracts is shown. The red line indicates the fast migrating band of Dot6, which corresponds to the hypo-phosphorylated form. (B) Cell growth is increased after glutamine and ammonium upshift, but not when using leucine or in cells treated with water. A representative growth curve of three independent replicates is shown. Increased phosphorylation of (C) Sch9 C-term and (D) Dot6 correlate with the N-source induced increased in cell growth.

Intracellular glutamine is a metabolic input to activate TORC1. As described above, prolonged stimulation of TORC1 by preferred N-sources correlates with increased growth rate. These data suggest that the transient TORC1 activation elicited in the first minutes of nutrient upshift, common to all N-sources tested, is not sufficient to maintain growth. We tested this hypothesis by titrating the concentration of glutamine used for the nutrient upshift and monitoring the effects on Sch9 phosphorylation and growth rate. Indeed, a lower final concentration of glutamine (5 mg/L, Gln 1%) was still able to rapidly induce TORC1 activity, but failed to sustain Sch9 phosphorylation over time (Fig. 9A and B). The low glutamine concentration also failed to enhance the growth rate of cells grown on proline, while an intermediate concentration of glutamine (50 mg/L, Gln 10%) only partially increased growth (Fig. 9C). In conclusion, low concentrations of glutamine, unable to sustain TORC1 activity over time and consequently growth, still trigger an early activation wave of TORC1.

The above data suggest two distinct mechanisms for how N-sources stimulate TORC1. The first mechanism, responsible for the rapid, transient activation of TORC1, is triggered by all N-sources independent of quality and is unable to sustain growth. The second mechanism, in response to good N-sources, mediates sustained TORC1 activity and growth. The sustained stimulation of TORC1 could be dependent on a 'metabolic input' that a poor N-source is not able to generate or maintain. To investigate the nature of a potential ‘metabolic input’, we measured the changes in relative levels of several metabolites by mass spectrometry, focusing on the first 10 minutes after nutrient upshift. We reasoned that if a common metabolic input exists, its relative level would increase/decrease within the first minutes after nutrient upshift, and upon reaching a certain threshold would sustain TORC1 activity and growth. We consistently found that the intracellular level of glutamine increased in cells stimulated with a preferred N-source such as ammonium, asparagine or glutamine itself (Fig. 9D, E, G and H). Interestingly, glutamine levels increased two fold already within two minutes after ammonium and asparagine addition, but remained below the two-fold threshold for up to 9 minutes after addition of leucine (Fig. 9F). When stimulating with a lower amount of glutamine (1%), we observed a corresponding smaller and transient increase of intracellular glutamine (Fig. 9H). These data suggest that intracellular glutamine may be the common metabolite required for TORC1 activation.

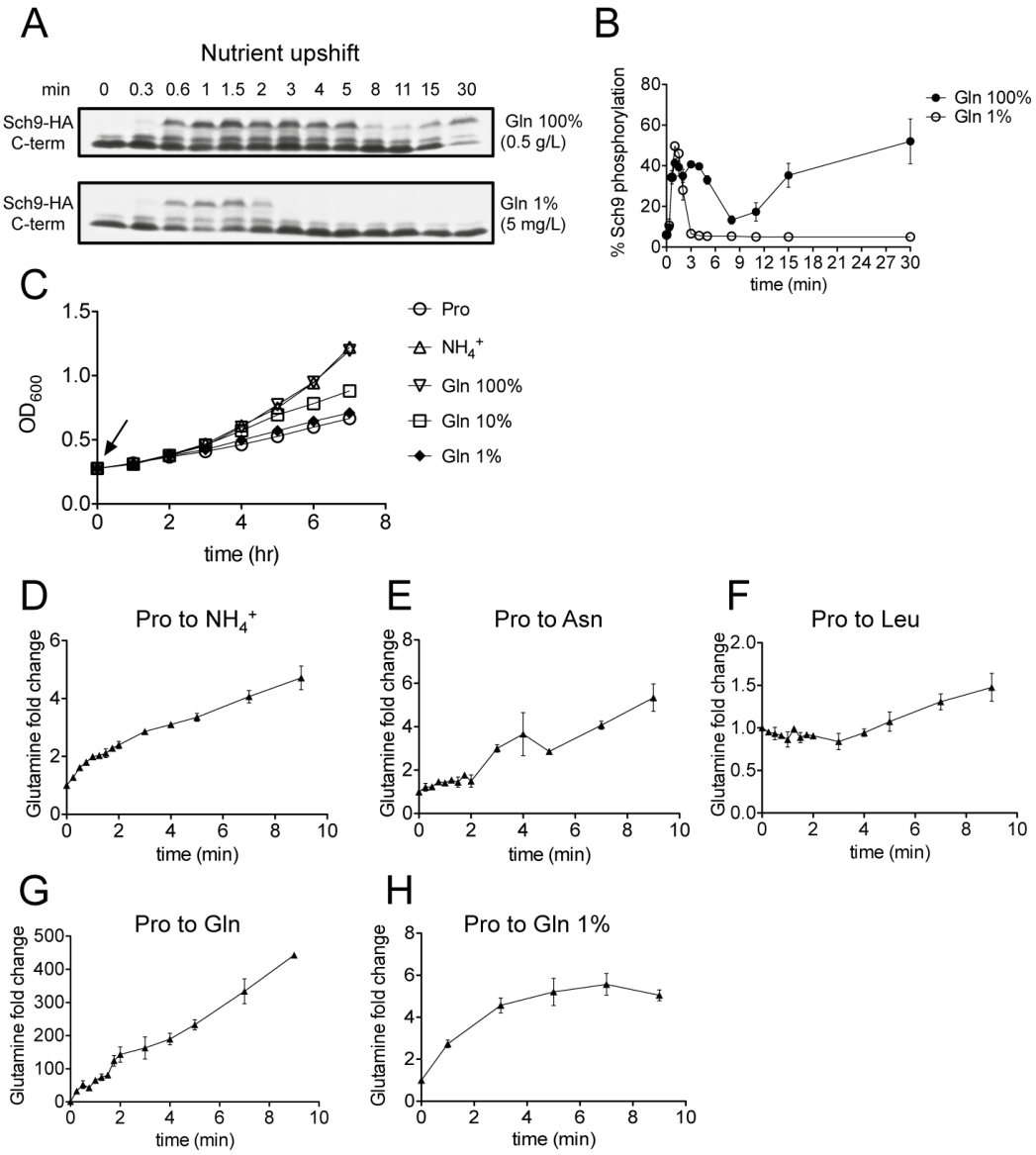

Figure 9: Intracellular glutamine constitutes a metabolic input to activate TORC1.

The increase in cell growth rate following nutrient upshift depends on the quality and quantity of the given N-source. (A and B) Nutrient upshift with 1% glutamine (final concentration 5 mg/L) results in low Sch9 C-term phosphorylation at 30 min after shift compared with nominal (100%) glutamine concentration (final concentration 0.5 g/L). Relative Sch9 C-term phosphorylation quantification is expressed as mean ± SEM. (C) Cell growth rate with different N-sources at different concentrations. Where not specified or when 100%, the final concentration of the N-source is 0.5 g/L. The black arrow indicates the time of stimulation. A representative growth curve of three independent replicates is shown.

Glutamine synthesis and accumulation within the first 10 minutes after nutrient upshift are key features of high-end, preferred N-sources. Intracellular glutamine levels measured by LC-MS/MS during nutrient upshifts with (D) ammonium, (E) asparagine, (F) leucine, (G) glutamine at the final concentration of 0.5 g/L (100%), and (H) glutamine at the final concentration of 5 mg/L (glutamine 1%). Fold changes of relative glutamine levels are expressed as mean ± SEM.

Next, we investigated the role of glutamine synthesis in TORC1 activation during nutrient upshift. To this end, we used the drug methionine sulfoximine (MSX), a specific inhibitor of glutamine synthetase. MSX treatment of cells growing in glutamine-free media leads to an arrest in cell growth, due to decreased intracellular glutamine levels and decreased TORC1 signaling (Crespo et al., 2002). As previously reported, MSX treatment compromised growth of cells grown in the presence of ammonium or leucine, but not in the presence of glutamine (data not shown). We tested the effect of blocking glutamine synthesis on activation of TORC1 by treating cells for one hour with MSX before addition of an N-source. Pre-treatment with MSX prevented Sch9 phosphorylation when stimulating with leucine or ammonium (Fig 10D and E.). When stimulating with ammonium, Sch9 phosphorylation was significantly impaired in both the initial and delayed phases of TORC1 activation ($p < 0.01$) (Fig. 10D). MSX decreased the transient stimulation observed with leucine ($p < 0.05$) (Fig. 10E). However, no significant difference in the increase in Sch9 phosphorylation was observed in cells stimulated with glutamine (Fig. 10F). These data suggest that ammonium assimilation into glutamine is essential for ammonium to be sensed and translated into the metabolic input activating TORC1. We conclude that the intracellular level of glutamine is important for TORC1 activation by the N-source. Intracellular accumulation of glutamine constitutes a metabolic input for TORC1 activation in response to a good N-source.

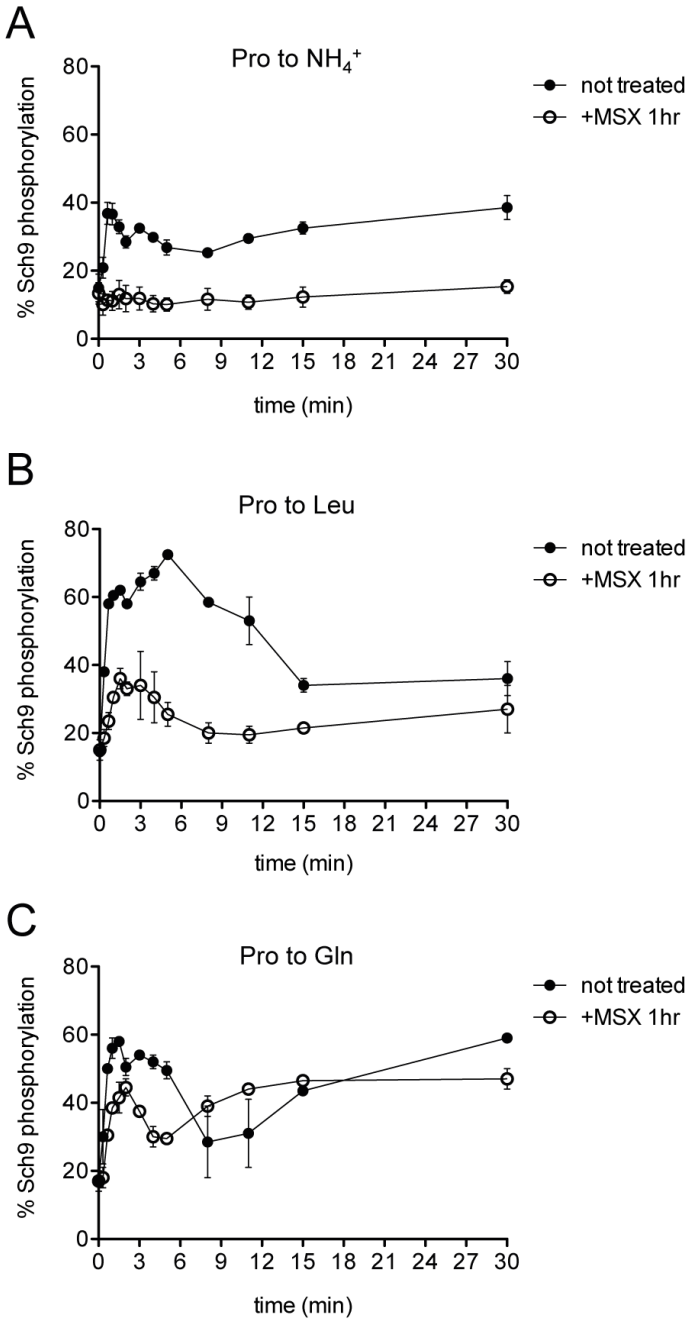

Figure 10: MSX treatment blocks the metabolic input to TORC1.
Glutamine synthesis and accumulation constitute a metabolic input signal to TORC1. MSX pre-treatment (2 mM, 1 h) reduces the Sch9 C-term phosphorylation increase during nutrient upshift with (A) ammonium and (B) leucine, but not when using (C) glutamine. Relative quantifications are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA.

The metabolic input acts independently of Gtr/Rag in TORC1 activation. Next, we addressed the mechanism by which the N-source activates TORC1, and how this is related to amino-acid-dependent activation of TORC1. Leucine and possibly other amino acids activate TORC1 via EGOC (see Introduction), whereas the mechanism of TORC1 activation by N-sources such as ammonium and glutamine is unknown. To address the role of EGOC in TORC1 signaling during N-source upshift, we examined glutamine stimulation in strains lacking *GTR1* or *VAM6*. Glutamine failed to stimulate rapid Sch9 phosphorylation in *gtr1Δ* and *vam6Δ* strains, but was still able to induce the delayed, sustained Sch9 phosphorylation (Fig. 11A and B). The delayed Sch9 phosphorylation in the deletion strains was comparable to that observed in a wild-type strain (Fig. 11A and B). We also tested the effect of inhibiting Gtr1 using 1,3-dihydro-1-hydroxy-2,1-benzoxa-borole (DHBB). DHBB occupies the editing site in LeuRS, inhibiting the interaction of LeuRS with Gtr1 and favoring hydrolysis of Gtr1-bound GTP (Bonfils et al., 2012). DHBB pre-treatment had no effect on Sch9 phosphorylation following glutamine stimulation, but decreased TORC1 activation upon stimulation with leucine (Fig. 11C, D, E and F). These data are consistent with a positive role for EGOC in the rapid, transient activation of TORC1, and at the same time suggest that glutamine accumulation is able to stimulate and sustain TORC1 activity even in the absence of a functional EGOC. Moreover, LeuRS appears to play a role in the specific activation of TORC1 by leucine, and is dispensable for the N-source input.

To investigate further the requirement of EGOC for growth, we compared growth of a wild-type and several EGOC mutant strains on solid YMM media supplemented with only one nitrogen source. We used *vam6Δ*, *gtr1Δ* and *gtr2Δ* strains and strains in which we complemented *GTR1* loss by ectopically expressing wild-type *GTR1* or allelic versions of *GTR1* encoding inactive GDP-bound (*GTR1*^{S20L}) or active GTP-bound Gtr1 (*GTR1*^{Q65L}). Strains lacking *GTR2* grew worse than other EGOC deficient strains (Fig. 11G), indicating a dominant role for *GTR2* over *GTR1* as previously reported (Binda et al., 2009). This may be due to a more prominent role of Gtr2 in permease sorting, although both *GTR2* and *GTR1* were reported to be required for the plasma membrane sorting of Gap1 and Put4 (Gao and Kaiser, 2006). Consistent with this explanation, we found that the *gtr2Δ* strain failed to grow on proline as the sole N-source. Conversely, the *gtr1Δ* strain was viable on proline medium (Fig. 11G), indicating that at least in our background (S288c derived prototroph) Gtr1 is not required for permease sorting to the plasma membrane. Deletion of *GTR1* negatively affected growth only when cells were forced to use leucine as the sole N-source. *GTR1* was dispensable for growth in the presence of the preferred N-source glutamine or ammonium (Fig. 11G). Expression of wild-type or constitutively active Gtr1 restored growth of *gtr1Δ*

cells on leucine. Expression of inactive GDP-bound Gtr1 further impaired growth on leucine, but had no remarkable effect when cells were grown in the presence of glutamine or ammonium (Fig. 11G). Also, growth of a *vam6Δ* strain was rescued in the presence of glutamine or ammonium. When grown on proline or leucine, a *vam6Δ* strain displayed a phenotype intermediate to that exhibited by *gtr1Δ* or *gtr2Δ* strains (Fig. 11G), suggesting a role for Vam6 in growth regulation in addition to its role as a GEF for Gtr1. We conclude that preferred N-sources, such as glutamine and ammonium, sustain growth independently of Gtr/Rag, while cells cultured in the presence of leucine as the only N-source require functional Gtr/Rag.

The EGOC components Gtr2, Ego1 and Ego3 were initially identified as necessary for restoration of growth after rapamycin treatment (Dubouloz et al., 2005). Indeed, we found that *vam6Δ*, *gtr1Δ* and *gtr2Δ* strains, independently of the N-source, were unable to resume growth following rapamycin treatment (Fig. 11G lower panel). In the *gtr1Δ* strain, growth was restored by expression of wild-type as well as constitutively active Gtr1. These data suggest that EGOC is essential for restoration of growth following rapamycin treatment, independent of the nutritional environment. Overall, we show that glutamine activates TORC1 independently of Gtr/Rag.

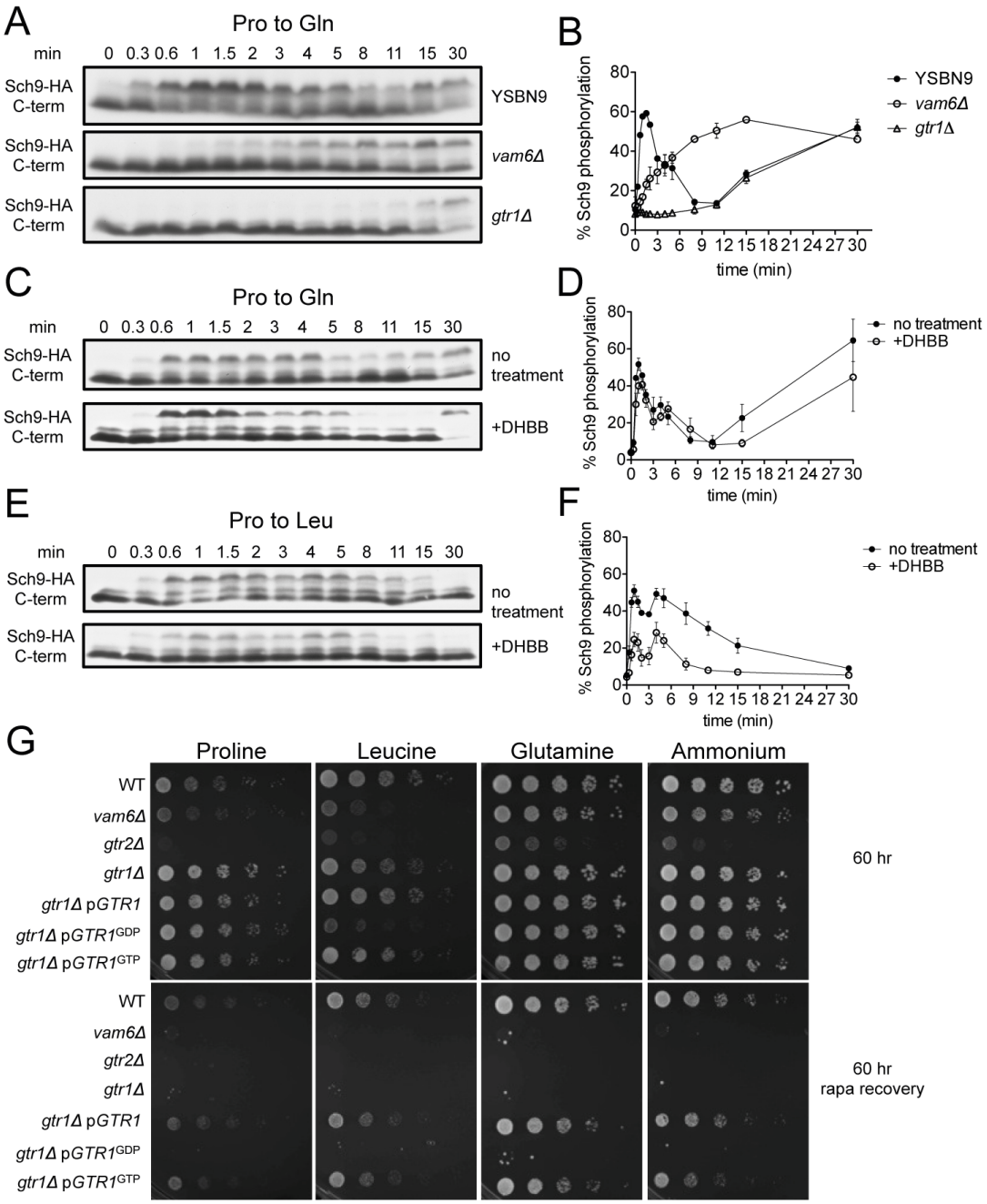

Figure 11: The metabolic input acts independently of the EGO complex in TORC1 activation.

(A and B) Following glutamine upshift, only the rapid, transient increase in Sch9 C-term phosphorylation is impaired in the *vam6Δ* and *gtr1Δ* strains compared to wild-type. DHBB pre-treatment (10 µM, 30 min) has no effect on Sch9 phosphorylation following (C and D) glutamine upshift, while it affects TORC1 activation in response to (E and F) leucine stimulation. Western blot of NTCB treated samples (panel A, C and E) and relative quantifications expressed as mean ± SEM (panel B, D and F).

(G) Growth assay on YMM solid media containing the indicated N-sources at the final concentration of 2.5 mg/mL. In the lower panel, cells were plated following rapamycin treatment (200 nM, 6 hours). All the indicated strains were initially grown in YMM supplemented with ammonium at the final concentration of 0.5 g/L and spotted on solid media following serial dilutions (1:5) starting at OD_{600} of 0.2.

2.5 DISCUSSION

Here, we investigated in vivo activation of TORC1 by N-sources and whether the underlying molecular mechanism requires Gtr/Rag. Both high (glutamine or ammonium) and low quality (leucine) N-sources stimulated a rapid, transient activation of TORC1 in a Gtr/Rag dependent manner. However, only high quality N-sources sustained TORC1 activity and growth. The preferred N-sources ultimately sustained TORC1 activity via glutamine synthesis and/or accumulation. Intracellular glutamine sustained TORC1 activity independently of Gtr/Rag. These findings suggest that nutrients activate TORC1 via different molecular mechanisms, and explain why Gtr/Rag is not essential for TORC1 signaling or viability in the presence of preferred N-sources.

Several observations support our suggestion that Gtr/Rag is not the only mechanism of TORC1 activation by nutrients. First, constitutively active GTP-bound Gtr1 is unable to sustain TORC1 activity following ammonium starvation (Binda et al., 2009). Second, mutations that likely increase the intracellular levels of glutamine suppress the inability of EGOC mutants to resume growth after rapamycin treatment (Dubouloz et al., 2005). Third, we show that glutamine but not leucine sustains growth of *vam6* and *gtr1* strains defective in EGOC function. Fourth, we also show that EGOC is required for an initial, transient stimulation of TORC1 in response to all examined nutrients, but is not required for sustained TORC1 signaling in response to a good N-source. Thus, a good N-source such as glutamine activates TORC1 via a mechanism independent of EGOC. The decrease in Sch9 phosphorylation between the transient and sustained peaks of TORC1 activity might reflect the alternate onset of the EGO- and the glutamine-dependent mechanisms of TORC1 activation. The mechanism by which glutamine activates TORC1 in the absence of EGOC remains to be determined.

In mammalian cells, two models have been proposed to explain how amino acids influence the GTP loading of Rags. In one model, amino acids stimulate RagA/B^{GTP} by a so-called lysosomal inside-out mechanism, dependent on the vacuolar ATPase but independent of a proton gradient (Zoncu et al., 2011). In a second model, glutamine and leucine stimulate glutaminolysis and thereby α-ketoglutarate production that enhances GTP loading of RagA/B which ultimately activates mTORC1 (Durán et al., 2013; 2012). Strikingly, in yeast, glutamine synthesis and accumulation, rather than glutaminolysis, positively regulate TORC1 activity. This may reflect the metabolic differences between mammalian cells and budding yeast. In proliferating mammalian cells, ATP is produced mainly via mitochondrial oxidative phosphorylation. In this case, the flux through the TCA cycle is required to replenish the NADH pool to maintain respiration. Thus, in mammalian cells, the equilibrium α-ketoglutarate ↔

glutamate ↔ glutamine is pushed to the left, requiring α-ketoglutarate to sustain high flux through the TCA cycle (Tennant et al., 2009). Conversely, exponentially growing *S. cerevisiae* provided with a fermentable sugar, favor glycolysis over respiration, even in aerobic conditions and even though glycolysis is less efficient at producing ATP (Broach, 2012; Gancedo, 1998). Glucose also affects the expression of genes involved in nitrogen metabolism, such as the NADP-dependent glutamate dehydrogenase encoding genes *GDH1* and *GDH3* (DeLuna, 2001; Ljungdahl and Daignan-Fornier, 2012). In the presence of glucose, *GDH1* is highly expressed and accounts for the reductive amination of α-ketoglutarate to glutamate. Thus, glutamate levels are kept high in the presence of glucose, but ammonium is then required to convert glutamate to glutamine. In this situation, ammonium is limiting for growth (Schure et al., 1995). Preferred N-sources such as ammonium or glutamine, by favoring glutamine synthesis or accumulation, provide a richer nutritional environment leading to increased TORC1 signaling and faster growth. Non-preferred N-sources, unable to drive glutamine synthesis and accumulation, result in lower TORC1 activation and slower growth.

2.6 MATERIAL AND METHODS

Yeast strains, plasmid and media. Yeast strains and plasmids used in this study are listed in Table 1 and 2. Yeast cultures were grown in flasks at 30°C in liquid YMM containing only one source of nitrogen, at a final concentration of 0.5 g/L. Detailed composition of 1 L YMM is as follows: 5 g $(NH_4)_2SO_4$; 3 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 1.5 mg EDTA; 4.5 mg $ZnSO_4 \cdot 7H_2O$; 0.3 mg $CoCl_2 \cdot 6H_2O$; 1 mg $MnCl_2 \cdot 4H_2O$; 0.3 mg $CuSO_4 \cdot 5H_2O$; 4.5 mg $CaCl_2 \cdot 2H_2O$; 3 mg $FeSO_4 \cdot 7H_2O$; 0.4 mg $NaMoO_4 \cdot 2H_2O$; 1 mg H_3BO_3; 0.1 mg KI; 5 µg biotin; 100 µg Ca-pantothenate; 100 µg nicotinic acid; 2.5 mg inositol; 100 µg pyridoxine; 20 µg *p*-aminobenzoic acid; 100 µg thiamine; 20 g/L glucose; 0.5 g/L N-source ; to 1 L with 100 mM KH-phthalate-water pH 5.

Growth conditions and cell extracts. Typically, cells were grown in YMM supplemented with the first N-source at a concentration of 0.5 g/L in shaking flasks at 30°C until mid log phase ($OD_{600} \approx 1.0$). In the case of nutrient upshift, the second N-source was added at time point 0 at the final concentration of 0.5 g/L directly in the media. When performing nutrient downshifts, cells were gently pelleted by spinning for 2 min at 1000 g and re-suspended, at time point 0, in fresh YMM media containing L-glutamine at the final concentration of 0.5 g/L. For each time point, 9 mL of culture were taken, quenched with cold trichloroacetic acid (10% final concentration), and incubated on ice for 5 min. Cells were then pelleted at 3,500 g for 5 min, washed twice with cold acetone and dried in a speed-vac. The cellular pellet was re-suspended in 100 µL urea buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS), and mixed with 200 µL of glass beads. Cell lysis was performed using a bead-beater (Biospec Products), beating 5 times for 45 seconds at max speed with 3 minutes intervals on ice. The extract was collected and heated to 65°C for 10 min. Unbroken cells and debris were removed by centrifugation at 13,000 g for 5 min. Protein concentration was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL USA).

Chemical fragmentation and phosphorylation quantification of Sch9. Cell extracts were subjected to NTCB cleavage as described previously (Urban et al., 2007). Further analysis was done by SDS-PAGE and immunoblotting using anti-HA antibody (HA-Tag (6E2) Mouse mAb #2367, Cell Signaling Technology, Danvers, MA, USA). To quantify Sch9 phosphorylation, we used the software ImageJ (Schneider et al., 2012). We measured the integrated density of the upper-most band (phosphorylated protein) and divided it by the sum of the integrated density of the upper-most plus the lower-most band (unphosphorylated protein).

Metabolite extraction. All samples were supplemented with 50 µL ^{13}C-labeled internal standard and extracted one time with 1 mL 75%(v/v) ethanol buffered with 10 mM ammonium acetate pH 7.5, at 78°C for 3 min. After the extraction step, biomass was separated by centrifugation for 3 minutes at 5000 rpm at -9°C. The liquid extract of each sample was dried at 10^{-1} mbar to complete dryness in a RapidVac and then stored at -80°C until re-suspension.

Metabolite mass spectrometry. Liquid chromatography separation of compounds was achieved by an ion pairing-reverse phase method developed for UHPL (ultra high performance liquid chromatography) Q3 systems (Buescher et al., 2010), based on previously published high pressure methods (Büscher et al., 2009; Ewald et al., 2009; Luo et al., 2007) and implemented on a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) using a Waters Acquity T3 end-capped reverse phase column with dimensions 150 x 2.1 mm x 1.8 µm (Waters Corporation). Selective and sensitive detection of compounds was achieved by coupling liquid chromatography to a Thermo TSQ Quantum Ultra QQQ mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA) using a heated electrospray ionization source. The mass spectrometer was operated in negative mode with selected reaction monitoring (SRM). Fragmentation parameters were optimized individually for all compounds (Buescher et al., 2010). Both acquisition and peak integration were performed with the Xcalibur software version 2.07 SP1 (Thermo Fisher Scientific). Peak areas were normalized to fully ^{13}C-labeled internal standards (Wu et al., 2005) and the amount of biomass.

2.7 TABLES

Table 1: Strains used in this study.

Strain	Genotype	Source
YSBN9	MAT*α FY3 ho::Ble ura3-52*	Canelas et al., 2010
YSBN9 *vam6Δ*	MAT*α FY3 ho::Ble ura3-52 VAM6::KanMX*	this study
YSBN9 *gtr1Δ*	MAT*α FY3 ho::Ble ura3-52 GTR1::KanMX*	this study
YSBN9 *gtr2Δ*	MAT*α FY3 ho::Ble ura3-52 GTR2::KanMX*	this study
YSBN9 *DOT6*-3HA	MAT*α FY3 ho::Ble ura3-52 DOT6*-3xHA*::KanMX*	this study

Table 2: Plasmids used in this study.

Plasmid	Vector; Insert	Source
pRS416	cen *URA3*	
pJU733	pRS416; cen *URA3 SCH9*-3xHA	Urban et al., 2007
pMB1393	YCplac33; cen *URA3* Tet-on-*GTR1*	Binda et al., 2009
pMB1394	YCplac33; cen *URA3* Tet-on-*GTR1*-Q65L	Binda et al., 2009
pMB1395	YCplac33; cen *URA3* Tet-on-*GTR1*-S20L	Binda et al., 2009

2.8 ADDITIONAL RESULTS

2.8.1 Ser711 and Thr737 are two major TORC1-dependent phosphorylation sites of Sch9

INTRODUCTION

In our study, we measured TORC1 activity following perturbation of the nitrogen source. Sch9 is a direct target of TORC1. Sch9 phosphorylation state is used as readout of TORC1 activity. For this purpose, we measured and quantified Sch9 C-terminus phosphorylation state following chemical cleavage with NTCB and Western blot. We also directly measured by mass spectrometry the phosphorylation of the six single phosphosites target of TORC1. Previous studies showed that only simultaneous mutation of at least five sites to alanine prevented the phosphorylation induced mobility shift of Sch9 C-terminus in nutrient rich conditions (Urban et al., 2007). In our study we identified in S711, T737 and S765 the phosphosites subjected the most to TORC1 regulation in vivo during nutrient upshift.

Here, we investigated whether substitution of these residues with the non-phosphorylatable alanine would have an impact on the migration pattern of Sch9 C-terminus following nutrient upshift. These data are still preliminary and will be the subject of further studies.

RESULTS

First of all we obtained the plasmids harboring the Sch9 alanine mutants by PCR-based in situ mutagenesis. We then reintroduce the plasmids in the YSBN9 strain. We tested the electrophoretic mobility shift induced by nutrient upshift with glutamine on cells expressing a mutated copy of Sch9. Single substitution of S711 or T737 with alanine did not lead to any significant alteration of Sch9 C-terminus phosphorylation (Fig. 12A, B and C). On the other hand, we observed no TORC1-induced increase in Sch9 C-terminus phosphorylation when increasing TORC1 activity with glutamine in the double mutant S711A/T737A (Fig. 12A and D). By comparing the levels of Sch9 phosphorylation, it is clear how single mutations of S711A and T737A only partially decrease Sch9 C-terminus phosphorylation by 10-20%, while the double alanine substitution S711A/T737A prevents the TORC1-induced phosphorylation increase (Fig. 12E). We conclude that S711 and T737 are two major TORC1-dependent phosphorylation sites on Sch9 C-terminus. Simultaneous absence of both sites dramatically decreases the TORC1-

induced increase in Sch9 phosphorylation following nutrient upshift. When singularly mutated, phosphorylation on one or the other site is still responsible for the electrophoretic mobility shift induced by TORC1-dependent phosphorylation.

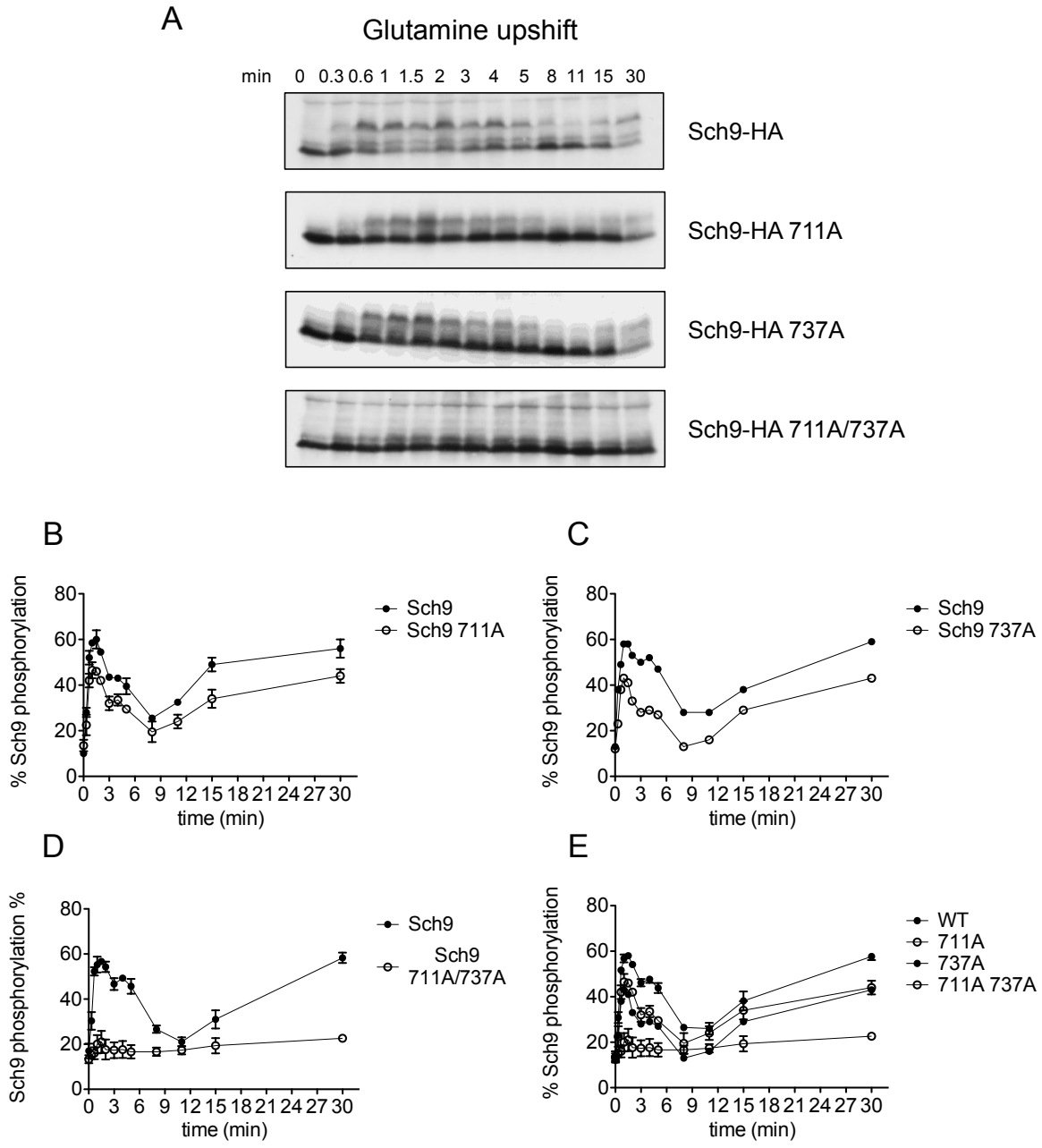

Figure 12: S711 and T737 are major TORC1-dependent phosphorylation sites of Sch9.
(A) Cells expressing different alanine mutated versions of *SCH9* were subjected to nutrient upshift with glutamine. Cell extracts were cleaved with NTCB and loaded on Western blot. (B, C, D and E) Quantification of signal obtained by Western blot.

DISCUSSION AND OUTLOOK

Six different residues on Sch9 C-terminus are phosphorylated in a TORC1 dependent manner. These sites were shown to be required for Sch9 activity in vitro and in vivo (Urban et al., 2007). We previously determined that the extent of phosphorylation of these sites is dependent on the quality of the nitrogen source. By mass spectrometry we identified three sites which phosphorylation state changes the most when TORC1 is activated by the quality of the nitrogen source: S711, T737 and S765. Simultaneous mutation of S711 and T737 to alanine blocks the phosphorylation increase of Sch9 C-terminus following nutrient upshift, indicating that these two sites are major TORC1 targets in vivo.

We further tested the impact of the alanine substitutions of Sch9 on growth. We used a strain deleted for *SCH9* expressing a plasmid harboring a wild-type or mutated version of *SCH9*. Despite a clear role as major TORC1 target sites in vivo, alanine substitution of S711 and T737 had no effect on growth in vivo (data not shown). Together with the observation that only the mutation to alanine of at least five residues on Sch9 C-terminus decreases Sch9 activity in vitro and in vivo (Urban et al., 2007), these data indicate that TORC1-dependent phosphorylation of any residue within Sch9 C-terminus can sustain Sch9 activity and growth. Or in alternative, another protein with a redundant function with Sch9, such as Sfp1 or Fhl1, could sustain ribosome biogenesis and growth when Sch9 activity is slightly compromised by interfering with Sch9 C-terminus phosphorylation.

Alanine substitution of S765, alone and in combination with S711 and T737, and its effect on Sch9 phosphorylation will be the subject of further studies.

MATERIALS AND METHODS

Sch9 immunoprecipitation and proteolysis

Cell lysis was performed using 1 mL of urea buffer and 1 mL glass beads starting from 90 mL of mid log-phase culture for each time point. After Pierce BCA protein quantification, same amounts of cell extracts were diluted 10-fold with breaking buffer (PBS 1x, 10% glycerol, 0.5% tween-20, PPi, and Pi) and pre-cleared with 50 µL of a 50% w/v suspension of Protein A Sepharose (…) for 90 minutes at 4°C rocking. From the pre-cleared extracts, SCH9-HA was immunoprecipitated overnight at 4°C rocking with 50 µL of a 50% w/v suspension of Protein A Sepharose beads covalently crosslinked with anti-HA antibody. The beads were then collected at 2000 rpm for 2 min, washed twice

with PBS, and three times with 100 mM Tris-HCl pH 8.0, 150 mM NaCl. After the final wash, beads were dried and diluted with 100mM Tris-HCl pH 8.0, 150mM NaCl to a final 50% w/v suspension. For proteolysis, immuno-precipitated SCH9-HA was digested with 250 ng endoprotenase LysC (Wako, Neuss, Germany) for 2 h at 37°C. The beads were then centrifuged at 1,500 g for 1 min and the supernatant was collected. One half of the endoproteinase LysC digest was acidified with TFA (1% final concentration), while the other half was further digested with 100 ng endoproteinase AspN (Roche, Rotkreuz, Switzerland) for 2 h at 37°C followed by a second aliquot of 100 ng endoproteinase AspN and overnight digestion at 37°C. The digestion was stopped adding TFA.

Protein mass spectrometric analysis

Digests were analyzed by capillary liquid chromatography tandem MS using a setup of a ProteoCol C18 trap column (0.15 x 10 mm, 3 µm particle size, 300 Å, SGE Analytical Science, Victoria, AU) and a separating (0.1 x 250 mm) column that had been packed with C18 ReproSil-Pur, 3 µm material (Dr. Maisch GmbH, Ammerbuch, Germany). The columns were connected online to an Orbitrap FT hybrid instrument (Thermo Scientific, Bremen, Germany). The solvents used for separation were 0.1% acetic acid/2% acetonitrile (solvent A) and 0.1% acetic acid/80% acetonitrile (solvent B). Peptides were injected via a 2 µL loop onto the trap column with the capillary pump of an Agilent 1200 (Agilent Technologies, Basel, Switzerland) system set to 4 µL/min. After 10 min, the trap column was switched into the flow path of the separating column. A linear gradient from 2 to 35% Solvent B in solvent A was delivered with an Agilent 1200 nano pump set to 300 nL/min. The eluting peptides were ionized at 1.7 kV. The mass spectrometer was operated in a data-dependent fashion. The precursor scan was done in the Orbitrap set to 30,000 resolution, while the fragment ions were mass analyzed in the LTQ part of the instrument. A top five method was run so that the five most intense precursor ions were selected for fragmentation. Singly charged ions were omitted from fragmentation and previously selected ions were dynamically excluded for 25 seconds. The normalized collision energy was set to 35% and for phosphopeptide analysis multistage activation was enabled. Automatic gain control was set to 500,000 and 10,000 for Orbitrap and LTQ, respectively.

Peptide identification

The LC/MS/MS data were analyzed with Proteome Discoverer 1.3 (Thermo Scientific, San José, CA, USA) against the SwissProt database. The Proteome Discoverer was set to use Mascot (version 2.4.0 (1)) as the search engine. A mass tolerance of 10 ppm was set. Cysteine carbamidomethylation was used as a fixed, and methionine oxidation, and phosphorylation on serine and threonine was set to variable modification. Two missed cleavages were allowed. For peptide identifications an FDR of ≤ 1% was set.

Strains and plasmids

A list of plasmids used for the experiments presented is given below in table 3.

For growth conditions, NTCB cleavage and signal quantification please refer to section 2.6, pages 58-59.

PCR-based site directed mutagenesis was performed as described before (Zheng et al., 2004).

Table 3

Plasmid	**Vector; Insert**	**Source**
pRS 416	cen *URA3*	
pJU 676	pRS 416; *SCH9*-5xHA	Urban et al., 2007
pDS 1	pRS 416; *SCH9 (S711A)*-5xHA	this study
pDS 2	pRS 416; *SCH9 (T737A*)-5xHA	this study
pDS 3	pRS 416; *SCH9 (S711A, T737A*)-5xHA	this study

3 APPENDIX

3.1 ACTIVATION OF mTORC2 BY ASSOCIATION WITH THE RIBOSOME

Zinzalla and colleagues made an important step forward in the understanding of TORC2 upstream regulation. The isolation of the *nip7-1* thermo-sensitive mutant in yeast led to the discovery that mTORC2 activation by growth factors requires TORC2 association with the ribosome. I helped in this work with the characterization of the yeast mutant *nip7-1*.

Cell

Activation of mTORC2 by Association with the Ribosome

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SUMMARY

The target of rapamycin (TOR) is a highly conserved protein kinase and a central controller of growth. Mammalian TOR complex 2 (mTORC2) regulates AGC kinase family members and is implicated in various disorders, including cancer and diabetes. Here, we investigated the upstream regulation of mTORC2. A genetic screen in yeast and subsequent studies in mammalian cells revealed that ribosomes, but not protein synthesis, are required for mTORC2 signaling. Active mTORC2 was physically associated with the ribosome, and insulin-stimulated PI3K signaling promoted mTORC2-ribosome binding, suggesting that ribosomes activate mTORC2 directly. Findings with melanoma and colon cancer cells suggest that mTORC2-ribosome association is important in oncogenic PI3K signaling. Thus, TORC2-ribosome interaction is a likely conserved mechanism of TORC2 activation that is physiologically relevant in both normal and cancer cells. As ribosome content determines growth capacity of a cell, this mechanism of TORC2 regulation ensures that TORC2 is active only in growing cells.

INTRODUCTION

Target of rapamycin (TOR) is a central controller of cell growth and metabolism in response to nutrients, growth factors, and energy status. TOR is found in two structurally and functionally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 (Wullschleger et al., 2006). The TOR complexes, originally described in yeast (Loewith et al., 2002), are conserved across all eukaryotes and regulate a wide spectrum of cellular processes that mediate cell growth (Laplante and Sabatini, 2009; Soulard et al., 2009; Wullschleger et al., 2006; Yang and Guan, 2007). In mammalian cells, mammalian TORC1 (mTORC1) contains mTOR, raptor, and mLST8 and is sensitive to the immunosuppressant and anticancer drug rapamycin (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). mTORC1 controls transcription, ribosome biogenesis, protein synthesis, lipid synthesis, nutrient transport, autophagy, and other growth-related processes. The best-characterized substrates of mTORC1 are S6K and 4E-BP via which mTORC1 controls translation (Sonenberg and Hinnebusch, 2009). mTORC2 consists of mTOR, rictor, mSIN1, mLST8, and PRR5/PRR5L (also known as protor1 and 2) and is insensitive to rapamycin, although long-term rapamycin treatment can indirectly inhibit mTORC2 in some cell types (Cybulski and Hall, 2009; Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2006; Sparks and Guertin, 2010). mTORC2 directly phosphorylates and activates the AGC kinases Akt (also known as PKB), SGK1, and likely PKC (Facchinetti et al., 2008; García-Martínez and Alessi, 2008; Hresko and Mueckler, 2005; Ikenoue et al., 2008; Jacinto and Lorberg, 2008; Sarbassov et al., 2004; Sarbassov et al., 2005). mTORC2 promotes cell survival via Akt and mediates organization of the actin cytoskeleton (Cybulski and Hall, 2009; Sparks and Guertin, 2010).

Both mTORC1 and mTORC2 are activated by growth factors, including insulin, IGF-1, and others. Growth factors activate mTORC1 via phosphatidylinositol 3-kinase (PI3K), PDK1, Akt, the TSC1-TSC2 complex, and Rheb, a small guanosine triphosphate (GTP)-binding protein that binds and activates mTORC1 directly (Avruch et al., 2009; Manning and Cantley, 2003). In contrast, the mechanism via which growth factors activate mTORC2 has been elusive. Growth factors activate mTORC2 via PI3K (Frias et al., 2006; García-Martínez and Alessi, 2008; Yang et al., 2006), but signaling steps beyond PI3K are distinct from those upstream of mTORC1 and unknown (Cybulski and Hall, 2009; Sparks and Guertin, 2010). The nature of the upstream regulators of TORC2 in unicellular model organisms such as yeasts, which lack a growth factor signaling pathway, is completely unknown (Soulard et al., 2009).

Here, we describe a genetic screen in yeast and subsequent studies in mammalian cells that identify the ribosome as an activator of TORC2. We demonstrate that the ribosome, independent of protein synthesis, is required for mTORC2 signaling in vivo and mTORC2 kinase activity in vitro. Active mTORC2 is associated with the ribosome. Insulin stimulates the association of mTORC2 with the ribosome via PI3K signaling. Findings with cancer cells suggest that ribosome-dependent mTORC2 activation is physiologically relevant in tumors with hyperactive PI3K signaling.

RESULTS

A Genetic Screen Reveals that NIP7 Is Required for TORC2 Signaling in Yeast

To identify upstream activators of TORC2, we performed a genetic screen in the budding yeast *S. cerevisiae*. Furthermore,

we assumed that whatever activates TORC2 in yeast would also be upstream of mTORC2 in mammals. In other words, we assumed that growth factor signaling was grafted onto a heretofore unknown ancestral input controlling TORC2 in unicellular yeast. This reasoning was supported by the fact that growth factor signaling was grafted onto the ancestral nutrient input in the case of TORC1.

mTORC2 phosphorylates the hydrophobic motif in the AGC kinase SGK1 and thereby activates SGK1. In yeast, TORC2 similarly phosphorylates and activates the SGK1 ortholog YPK2. Our genetic screen was based on the observation that overexpression of constitutively active YPK2 ($YPK2^{D239A}$, hereafter referred to as YPK2*) suppresses the lethality of a TORC2 defect (Aronova et al., 2008; Kamada et al., 2005). Assuming that YPK2* would also suppress lethality caused by a defect in an upstream activator of TORC2, we performed a so-called reverse suppressor screen to isolate yeast mutants that depend on YPK2* for viability (Figure 1A and Experimental Procedures). This is referred to as a reverse suppressor screen because it starts with a suppressor mutation (YPK2*) to identify unknown "suppressee" mutations—the reverse order of a normal (forward) suppressor screen. The screen was predicted to identify loss-of-function mutations in an essential upstream activator of TORC2 or in the essential components of TORC2, including TOR2, AVO1 (mSIN1 ortholog), and AVO3 (rictor ortholog). The screen yielded a total of 44 independent mutants defective in TORC2, thereby validating the screen. The 44 mutants consisted of 25, 13, and 6 *tor2*, *avo1*, and *avo3* mutants, respectively. In addition, we obtained a temperature-sensitive *nip7* mutant that we hereafter refer to as *nip7-1* (Figure 1A and Figures S1A and S1B). Sequence analysis of the *nip7-1* allele identified a point mutation that converts glycine 71 to aspartic acid. Western blot analysis of extracts from *nip7-1* cells showed that the point mutation mildly and strongly decreased NIP7 protein levels at permissive temperature (25°C) and nonpermissive temperature (37°C), respectively (Figure S1A). YPK2*, but not wild-type YPK2, suppressed the growth defect of the *nip7-1* mutant at semipermissive temperature (30°C and 34°C) (Figure S1B). NIP7 is an essential protein required for maturation of rRNA of the 60S ribosomal subunit (Zanchin et al., 1997). Confirming this role of NIP7 in ribosome biogenesis, we observed a reduction in the amounts of the 60S subunit, the 80S ribosome, and polysomes, with a concomitant appearance of halfmer polysomes, in extracts of the *nip7-1* mutant grown at semipermissive temperature (30°C) (Figure S1C).

To examine further whether NIP7 is required for TORC2 signaling, we investigated whether the *nip7-1* mutant phenocopied TORC2 mutants. The *nip7-1* mutant indeed exhibited several defects similar to those observed in temperature-sensitive TORC2 mutants (*avo3-1* and *tor2-21*) (Aronova et al., 2008; Beeler et al., 1998; Facchinetti et al., 2008; Helliwell et al., 1998; Kamada et al., 2005; Schmidt et al., 1997). First, the *nip7-1* mutant exhibited reduced signaling through the cell wall integrity pathway, as evidenced by decreased MPK1 phosphorylation and PKC1 protein levels, depolarization of the actin cytoskeleton, and restoration of growth in the presence of the osmotic stabilizer sorbitol (Figure 1B and Figure S1D). Second, the *nip7-1* mutant showed impaired sphingolipid biosynthesis, including hypersensitivity to myriocin, an inhibitor of the first step in the sphingolipid biosynthetic pathway, and restoration of growth in the presence of Ca^{2+} in a *csg2Δ* background (Figure S1E). Third, the *nip7-1* mutant showed decreased YPK2 kinase activity, as measured by an in vitro kinase assay with immunopurified YPK2 and, as a control, kinase-dead $YPK2^{K373A}$ (Figure 1C and data not shown for kinase dead). Finally and most importantly, TORC2 kinase activity was reduced in the *nip7-1* mutant, as measured by an in vitro kinase assay with TORC2 immunopurified from wild-type cells and *nip7-1* mutant cells grown at 30°C (Figure 1D). The above results strongly suggest that NIP7 is required, directly or indirectly, for TORC2 kinase activity and signaling.

mNIP7 Is Required for mTORC2 Activity and Ribosome Maturation in Mammals

Yeast NIP7 shares 75% identity with an uncharacterized mammalian protein also termed NIP7 (Figure S2A). We examined whether mammalian NIP7 (mNIP7) is required for mTORC2 signaling. mTORC2 directly phosphorylates Ser473 in the hydrophobic motif of Akt and Ser422 in the hydrophobic motif of SGK1 and thereby activates Akt and SGK1 toward substrates such as FoxO3a (Thr32) and NDRG1 (Thr346), respectively (García-Martínez and Alessi, 2008; Sarbassov et al., 2005). mTORC2 also autophosphorylates sites in rictor (Sarbassov et al., 2004; Jacinto et al., 2004) and is required for phosphorylation of Thr450 in the turn motif of Akt (Facchinetti et al., 2008; Ikenoue et al., 2008). Knockdown of mNIP7 in HeLa and HEK293 cells strongly decreased basal and insulin-stimulated phosphorylation of Ser473 and Thr450 in Akt, Thr32 in FoxO3a, Thr346 in NDRG1, and rictor in mTORC2 (Figure 2A, Figure S2B, and data not shown). mNIP7 knockdown had no effect on Erk phosphorylation or mTORC1-dependent S6K phosphorylation (Figure 2A). These findings suggest that mNIP7 is specifically required for mTORC2 signaling. Finally, we observed that mNIP7 knockdown reduced (by 58%) mTORC2 kinase activity in vitro, with no effect on mTORC2 amount or integrity (Figure 2B). These findings indicate that mNIP7 is required for mTORC2 activity and signaling, and not for mTORC2 synthesis, assembly, or stability. Furthermore, NIP7 is required for TORC2 signaling in both yeast and mammalian cells, suggesting a conserved mechanism of TORC2 activation.

Is mNIP7 a 60S maturation factor like NIP7 in yeast? Knockdown of mNIP7 reduced the amounts of the 60S ribosomal subunit and the 80S ribosome (80S), with no effect on the amount of the 40S subunit (Figure S2C). Knockdown of mTORC2 component mSIN1 had no effect on the ribosome profile (Figure S2C), indicating that the effect of mNIP7 on ribosome maturation was not due to a defect in signaling downstream of mTORC2. Thus, NIP7 is conserved from yeast to human as a 60S ribosome maturation factor and a TORC2 activator.

Ribosomes, but Not Protein Synthesis, Are Required for mTORC2 Signaling

Does mNIP7 control mTORC2 via its role in ribosome maturation? To address this question, we examined whether ribosome content affects mTORC2 signaling. Knockdown of ribosomal protein Rpl7 (60S subunit) reduced the amounts of the 60S

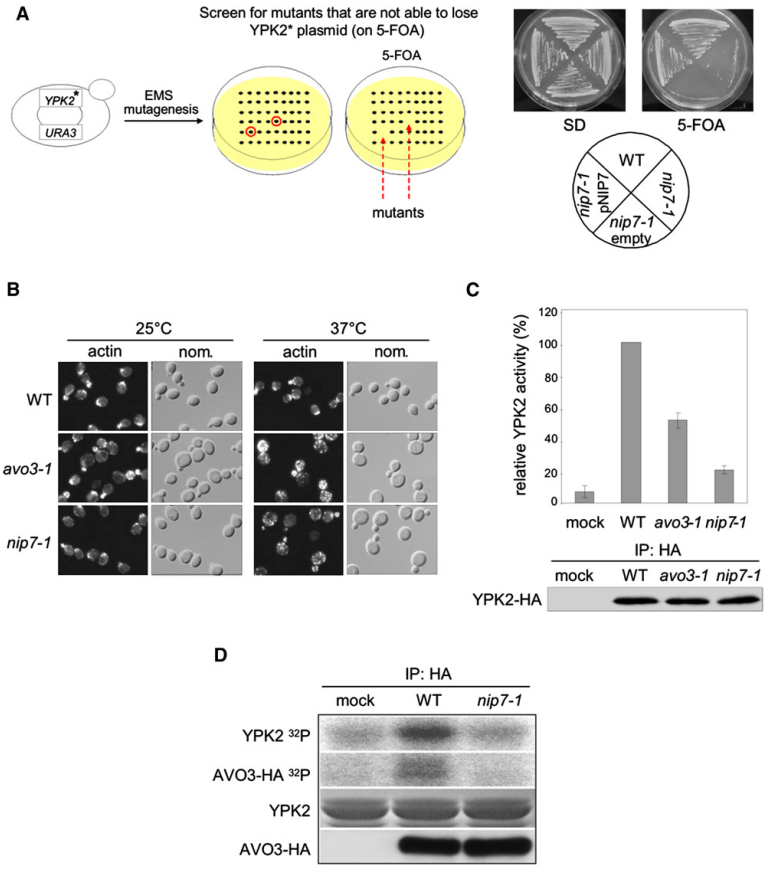

Figure 1. NIP7, Identified by a Reverse Suppressor Screen, Is Required for TORC2 Activity In Vivo and In Vitro

(A) Schematic representation of the reverse suppressor screen. A wild-type strain (JK9-3d**a**) overexpressing constitutively active YPK2* (YPK2^{D239A}) on an *URA3*-based plasmid was mutagenized with ethyl-methanesulfonate (EMS). Mutants that could not grow on plates containing 5-FOA were chosen for further analysis. A plasmid-borne *NIP7* gene (pNIP7) was isolated by complementation of the *nip7-1* mutation.

(B) *nip7-1* mutant exhibits depolarization of actin cytoskeleton. Wild-type (JK9-3d**a**), *avo3-1* (BAS65-2a), and *nip7-1* (DS1) cells grown in YPD at 25°C were shifted to 37°C for 6 hr. The actin cytoskeleton was stained with rhodamine-coupled phalloidin. A representative figure of three independent experiments for each strain is shown.

(C) *nip7-1* mutant shows decreased YPK2 activity. Wild-type cells (JK9-3d**a**) (mock) and wild-type, *avo3-1*, or *nip7-1* cells expressing plasmid-borne YPK2-HA (pYPK2-HA) were grown in YPD at 30°C. YPK2-HA was immunoprecipitated and subjected to in vitro kinase assay using cross-tide as a substrate. Substrate phosphorylation was quantified, and the average ± standard deviation from the mean based on three independent experiments is shown. Immunoprecipitated YPK2-HA was detected by western blotting (bottom).

(D) *nip7-1* mutant shows decreased TORC2 kinase activity in vitro. Wild-type cells (JK9-3d**a**; mock) and wild-type (DS2; WT) or *nip7-1* (DS3; *nip7-1*) cells expressing AVO3-HA were grown in YPD at 30°C. AVO3-HA was immunoprecipitated and subjected to a TORC2 kinase assay in vitro using recombinant YPK2 protein as a substrate. Also shown are Coomassie blue-stained total YPK2 protein and immunoblot of immunoprecipitated AVO3-HA.

See also Figure S1.

subunit and assembled ribosomes (80S monosomes and polysomes), whereas knockdown of ribosomal protein Rps16 (40S subunit) reduced the amounts of the 40S subunit and assembled ribosomes (Figure S2C). We also observed that knockdown of Rpl7 or Rps16 reduced the amounts of other proteins in the corresponding ribosomal subunit (Figure 3A and Figure S3A), consistent with the published finding that ribosomal subunit assembly determines the level of ribosomal proteins (Idol et al., 2007). Like knockdown of mNIP7, knockdown of either Rpl7 or Rps16 decreased basal and insulin-stimulated

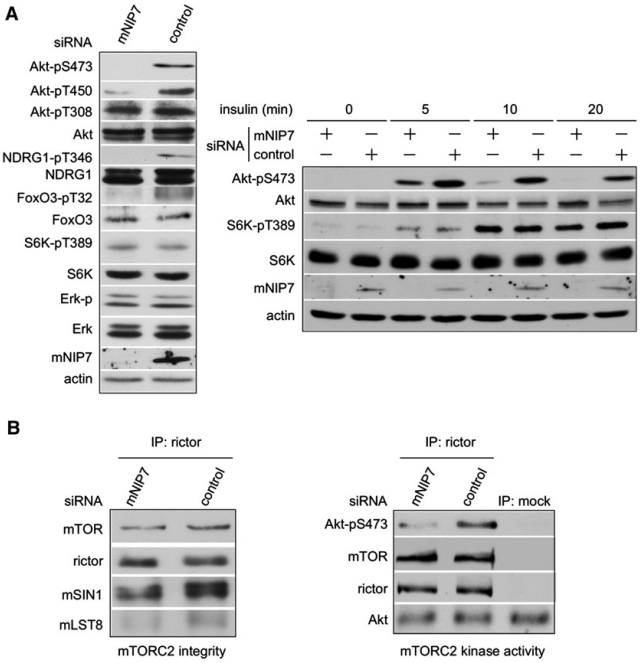

Figure 2. mNIP7 Is Required for mTORC2 Activity In Vivo and In Vitro

(A) siRNA-mediated knockdown of mNIP7 inhibits mTORC2 signaling. HeLa cells, 48 hr after transfection with the indicated siRNA, were harvested (left) or serum starved for 3 hr and then restimulated with insulin for the indicated times before harvesting (right). Phosphorylation and protein levels were determined by immunoblotting with the appropriate antibodies, as indicated.

(B) Knockdown of mNIP7 inhibits mTORC2 kinase activity toward Akt with no effect on mTORC2 integrity. rictor immunoprecipitates were immunoblotted with the indicated antibodies to determine mTORC2 integrity. mTORC2 in vitro kinase assay was performed using immunopurified mTORC2 (rictor) and recombinant, kinase-dead Akt as a substrate.

See also Figure S2.

phosphorylation of Akt (Ser473 and Thr450), FoxO3a (Thr32), NDRG1 (Thr346), and rictor, with no effect on Erk or S6K phosphorylation (Figure 3A, Figure S2B, and Figure S3A). Finally, we observed that Rpl7 knockdown reduced mTORC2 kinase activity, as determined by an in vitro kinase assay with immunopurified mTORC2 and recombinant, kinase-dead Akt as a substrate. Rpl7 knockdown had no effect on mTORC2 amount or integrity, as determined by unaffected coimmunoprecipitation of mTOR, mSIN1, or mLST8 with rictor (Figure 3B). Thus, assembled ribosomes (not 60S or 40S ribosomal subunits alone) are required for mTORC2 kinase activity and signaling. Furthermore, the above findings suggest that mNIP7 controls mTORC2 via its role in ribosome maturation.

mTORC2 promotes cell survival via phosphorylation and activation of Akt, which in turn phosphorylates and inhibits proapoptotic Bad (Brazil et al., 2004; Datta et al., 1997; Jacinto et al., 2006; Yang et al., 2006). To investigate further the physiological relevance of ribosome-mediated mTORC2 regulation, we examined the effect of mNIP7, Rpl7, or Rps16 knockdown on induction of apoptosis by etoposide or hydrogen peroxide (H_2O_2). Both treatments induce cell death in an Akt-sensitive manner (He et al., 2010; Kim et al., 2001; Wang et al., 2000). Knockdown of mNIP7, Rpl7, or Rps16 enhanced the induction of apoptosis by etoposide or hydrogen peroxide, as indicated by an increase in caspase 3 and PARP cleavage and a decrease in cell viability (Figure 4A and Figure S4). Knockdown of mSIN1 (mTORC2) similarly enhanced apoptosis. Knockdown of Bad blocked the proapoptotic effect of mSIN1, mNIP7, Rpl7, or Rps16 knockdown (Figure 4B). Thus, ribosomes appear to regulate mTORC2 signaling in a physiologically relevant manner.

Although ribosomal knockdown reduces mTORC2 kinase activity and signaling without affecting mTORC2 synthesis, is the process of protein synthesis per se required for mTORC2 activation? To address this question, we examined whether the protein synthesis inhibitors salubrinal, cycloheximide, anisomycin, or puromycin acutely inhibit mTORC2 signaling. Salubrinal blocks translation initiation by selective inhibition of eIF2α dephosphorylation (Boyce et al., 2005; Cnop et al., 2007) (Figure S3B). Cycloheximide, anisomycin, and puromycin inhibit translation elongation. Unlike knockdown of mNIP7, Rpl7, or Rps16, salubrinal, cycloheximide, anisomycin, and puromycin had no effect on basal or insulin-stimulated Akt Ser473 phosphorylation (Figures S3B–S3D and Figure S5C). All drugs were used under conditions known to affect protein synthesis in the expected manner, as determined by polysome gradient analysis (Figures S3B–S3D and data not shown). Thus, ribosomes mediate mTORC2 signaling independently of protein synthesis.

Active mTORC2 Is Associated with the Ribosome

To investigate the molecular mechanism by which ribosomes activate mTORC2 signaling, we examined by four unrelated methods whether ribosomes and mTORC2 physically interact. First, we determined whether mTORC2 coimmunoprecipitates with ribosomal protein Rpl26. Endogenous Rpl26 coimmunoprecipitated with endogenous mTOR, rictor, mSIN1, and ribosomal protein Rpl7 (Figure 5A), but not with raptor. This suggests that mTORC2, but not mTORC1, associates with the ribosome. Second, we determined whether mTORC2 copurifies with total ribosomes isolated by sedimentation through a sucrose cushion (see Experimental Procedures). mTOR and rictor, but not raptor, cosedimented with Rpl26, Rpl7, and Rps16 (Figure 5B). Third, although ribosomes activate mTORC2 independently of protein synthesis, we also determined whether mTORC2 cosediments

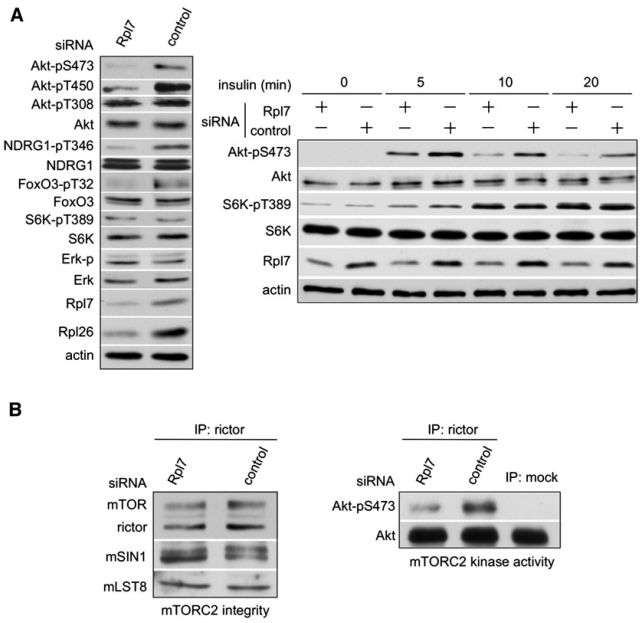

Figure 3. Ribosomes Are Required for mTORC2 Activity and Signaling

(A) siRNA-mediated knockdown of Rpl7 inhibits mTORC2 signaling. HeLa cells, 24 hr after transfection with the indicated siRNA, were harvested (left, basal activity) or serum starved for 3 hr and then restimulated with insulin for the indicated times before harvesting (right, insulin-stimulated activity). Phosphorylation and protein levels were determined by immunoblotting with the appropriate antibodies, as indicated.

(B) Knockdown of Rpl7 inhibits mTORC2 kinase activity toward Akt with no effect on mTORC2 integrity. rictor immunoprecipitates were immunoblotted with the indicated antibodies. mTORC2 in vitro kinase assay was performed using immunopurified mTORC2 (rictor) and recombinant, kinase-dead Akt as a substrate.

See also Figure S3.

with polysomes in a sucrose gradient. Lysates were fractionated in a sucrose gradient to separate polysomes from 80S, 60S, and 40S ribosomes. mTOR, rictor, and mSIN1 were found in both the polysomal and ribosomal fractions, to the same extent as Rpl7, Rpl26, and Rps16 (Figure S5A). Finally, we determined whether mTORC2 copurifies with mRNA-bound ribosomes. mRNA-bound ribosomes were purified by pull-down of poly(A) mRNA with oligo(dT) cellulose (Figure S5B) (Ceci et al., 2003). In this experiment, mTOR and rictor copurified with Rpl26, Rpl7, and the 40S ribosomal protein RACK1. RNase A treatment of a lysate before pull-down prevented isolation of any of the above proteins (Figure S5B), confirming interaction of mTORC2 with mRNA-bound ribosomes. The above data taken together suggest that mTORC2 physically interacts with translating (mRNA-bound) and nontranslating 80S ribosomes. An interaction between mTORC2 and the 80S ribosome is also supported by published mass spectrometry studies that identified several large and small subunit ribosomal proteins in mTORC2 immunoprecipitates (Pearce et al., 2007; Thedieck et al., 2007). We also note that nonionic detergent was required during any of our four above ribosome purifications to detect copurification of mTORC2 and that protein synthesis inhibitors had no effect on the interaction between mTORC2 and Rpl26 (Figure S5C).

To obtain more insight into the mTORC2-ribosome interaction, we performed coimmunoprecipitation experiments after knockdown of mTORC2 subunits or ribosomal proteins. First, the amount of mTOR in Rpl26 immunoprecipitates from rictor or mSIN1 knockdown cells was significantly reduced compared to control cells (Figure S6A), suggesting that mTORC2 interacts with the ribosome via rictor and/or mSIN1. We could not distinguish a requirement specifically for rictor or mSIN1 because knockdown of either rictor or mSIN1 alone also results in loss of the other. Second, knockdown of Rpl7 (60S subunit) abolished the interaction between mTORC2 and Rpl26, whereas knockdown of Rps16 (40S subunit) only moderately decreased the interaction between mTORC2 and Rpl26 (60S subunit) (Figure S6B). These results suggest that mTORC2 associates with the ribosome via rictor and/or mSIN1 binding to the 60S subunit.

The above findings suggest that ribosomes bind and activate mTORC2. To test this model further, we investigated whether ribosome-bound mTORC2 is indeed active. We performed an mTORC2 kinase assay with ribosomes purified either by Rpl26 immunoprecipitation or by sedimentation through a sucrose cushion, as described above. In both cases, a ribosome-associated kinase phosphorylated Ser473 in recombinant, kinase-dead Akt (Figure 5C). The mTOR inhibitor PP242 (Feldman et al., 2009) abolished the in vitro phosphorylation of Ser473 (Figure 5C), confirming that the ribosome-associated Ser473 kinase was mTORC2. Thus, ribosome-associated mTORC2 is active. Furthermore, we analyzed whether mTORC2 kinase activity is required for mTORC2-ribosome interaction. Treatment of HeLa cells with PP242 abolished Ser473 phosphorylation, as expected, but had no effect on mTORC2-Rpl26 interaction (Figure S6D), suggesting that mTORC2 activity is not required for mTORC2-ribosome association.

Insulin-PI3K Signaling Stimulates mTORC2-Ribosome Association

We next investigated whether mTORC2 association with the ribosome is regulated by insulin. Insulin treatment of serum-starved cells significantly increased the amount of mTOR and rictor that coimmunoprecipitated with Rpl26 (Figure 6A). Importantly, this stimulation of the interaction between mTORC2 and Rpl26 correlated with phosphorylation of the mTORC2 target site Ser473 in endogenous Akt. Thus, insulin stimulates mTORC2-ribosome association.

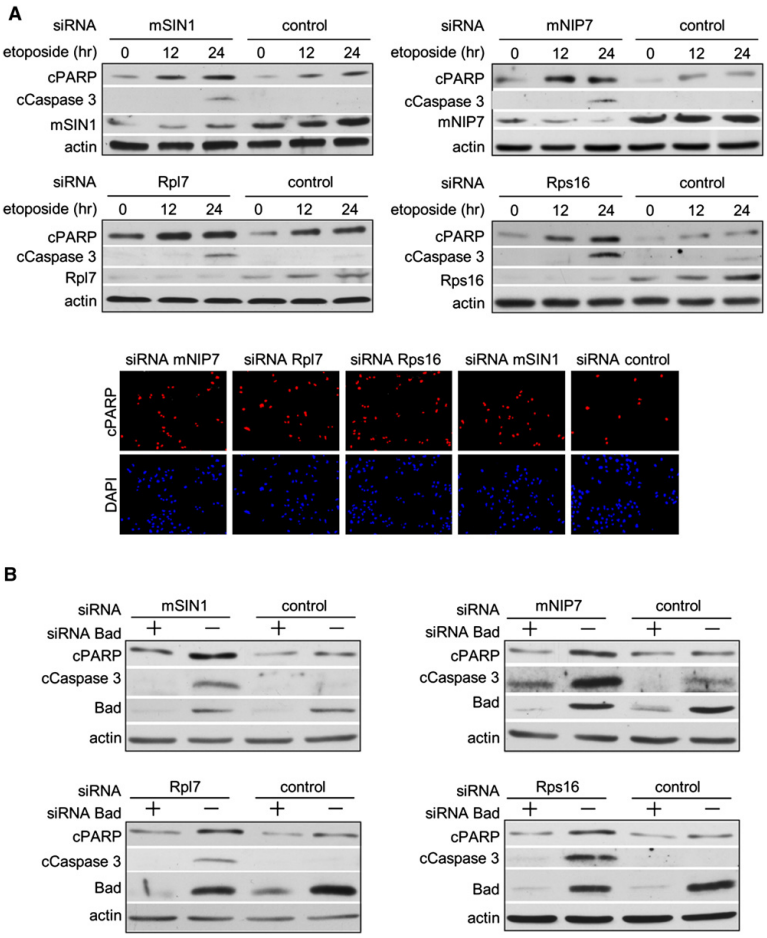

Figure 4. Ribosome or mTORC2 Knockdown Enhances Stress-Induced, Bad-Dependent Apoptosis

(A) siRNA-mediated knockdown of mSIN1, mNIP7, Rpl7, or Rps16 increases etoposide-induced apoptosis. HeLa cells were transfected with the various siRNAs and then treated with 25 μM etoposide for the indicated times (top) or for 24 hr (bottom). Extracts were analyzed by immunoblotting to assess efficiency of siRNA knockdown and induction of apoptosis with the indicated antibodies (top). Apoptosis was assessed by blotting for cleaved PARP (cPARP) and cleaved caspase 3 (cCaspase 3). Cells were fixed and stained with DAPI and cleaved PARP antibody to detect apoptotic cells (bottom).

(B) Bad is required for enhanced apoptosis in cells with knockdown of mSIN1, mNIP7, Rpl7, or Rps16. HeLa cells were transfected with the indicated siRNA and then treated with 25 μM etoposide for 24 hr. Extracts were analyzed by immunoblotting to assess the induction of apoptosis with cleaved PARP antibody. The efficiency of Bad knockdown is shown.

See also Figure S4.

Insulin activates mTORC2 via PI3K (Frias et al., 2006; García-Martínez and Alessi, 2008; Yang et al., 2006). To investigate whether PI3K signaling regulates mTORC2 association with the ribosome, we examined whether the mTORC2-Rpl26 interaction was affected upon inhibition or hyperactivation of PI3K signaling. Inhibition of PI3K strongly decreased mTORC2-Rpl26 interaction, as determined by a reduction in the amount of mTOR and rictor that coimmunoprecipitated with Rpl26 in lysates from cells treated with the PI3K inhibitor LY294002 (Figure 6B and Figure S6D). In contrast, hyperactivation of PI3K signaling by knockdown of PTEN, a negative regulator of PI3K signaling, increased the amount of mTOR and rictor in Rpl26 immunoprecipitates, compared to control cells (Figure 6C). Again, the change in mTORC2-Rpl26 interaction upon PI3K inhibition or hyperactivation was paralleled by a corresponding change in phosphorylation of the mTORC2 target site Ser473

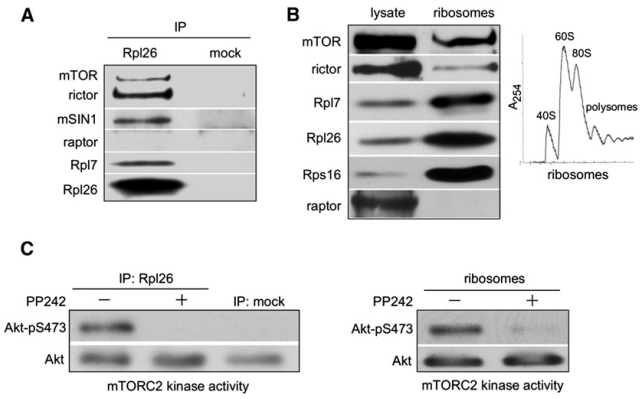

Figure 5. Active mTORC2 Is Associated with the Ribosome

(A) Endogenous Rpl26 coimmunoprecipitates with endogenous mTOR, rictor, and mSIN1. Rpl26 and mock immunoprecipitations were performed with HeLa cell extracts and analyzed by immunoblotting with the indicated antibodies.

(B) mTORC2 associates with ribosomes. Ribosomes were purified from HeLa cells by sedimentation through a sucrose cushion. Ribosomes were probed with the indicated antibodies (left) or were resedimented through a sucrose gradient to monitor 40S, 60S, and 80S ribosomes and polysomes. The absorbance profile of the sucrose gradient was determined at 254nm (right).

(C) Ribosome-associated mTORC2 is active. mTORC2 kinase assays were performed with Rpl26 or mock immunoprecipitates using recombinant, kinase-dead Akt as a substrate and in the presence or absence of the mTOR inhibitor PP242 (left). mTORC2 kinase assay was performed with ribosomes purified as described in (B), with recombinant, kinase-dead Akt as a substrate and in the presence or absence of PP242 (right).

See also Figure S5.

in endogenous Akt (Figures 6B and 6C). Taken together, the above data suggest that insulin stimulates mTORC2-ribosome association in a physiologically relevant manner via PI3K.

mTORC2-Ribosome Interaction Promotes Akt Signaling in Cancer Cells

PI3K-dependent association of mTORC2 with the ribosome, ribosome-mediated mTORC2 activation, and the physiological relevance of mTORC2-ribosome interaction in promoting cell survival prompted us to analyze mTORC2-ribosome association in cancer cells with hyperactive PI3K signaling. First, we examined whether PTEN-deficient metastatic melanoma cells have elevated mTORC2 activity. Approximately 60% of metastatic melanomas have reduced PTEN expression and elevated Akt phosphorylation (Robertson, 2005; Stahl et al., 2004). We analyzed PTEN expression and Akt Ser473 phosphorylation in

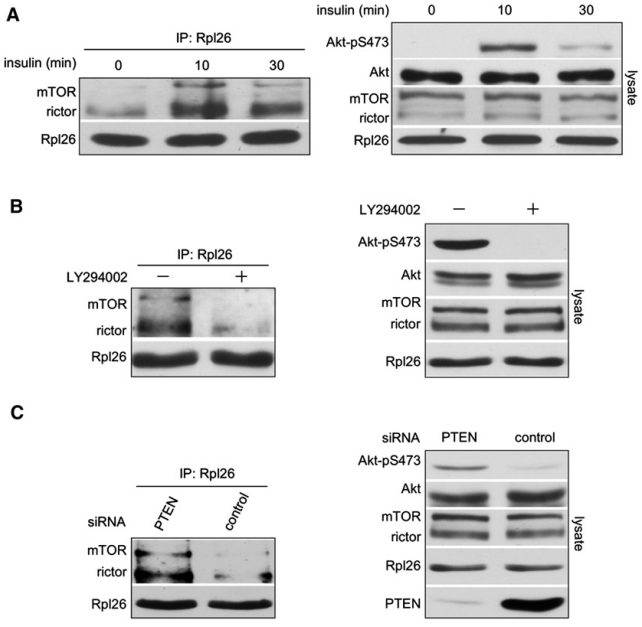

Figure 6. Insulin-PI3K Signaling Stimulates mTORC2-Ribosome Association

(A) Insulin stimulates mTORC2-ribosome association. HeLa cells were serum starved and then restimulated with insulin for the indicated time. Rpl26 immunoprecipitates (IP: Rpl26) and cell extracts (lysate) were immunoblotted with the indicated antibodies.

(B) PI3K inhibition decreases mTORC2-ribosome association. HeLa cells were treated with LY294002 (50 μM final concentration) for 30 min before harvesting. Rpl26 immunoprecipitates and cells extracts (lysate) were immunoblotted with the indicated antibodies.

(C) siRNA-mediated knockdown of PTEN increases mTORC2-ribosome association. HeLa cells were transfected with the indicated siRNA and then harvested after 48 hr. Rpl26 immunoprecipitates and cell extracts (lysate) were analyzed by immunoblotting with the indicated antibodies.

See also Figure S6.

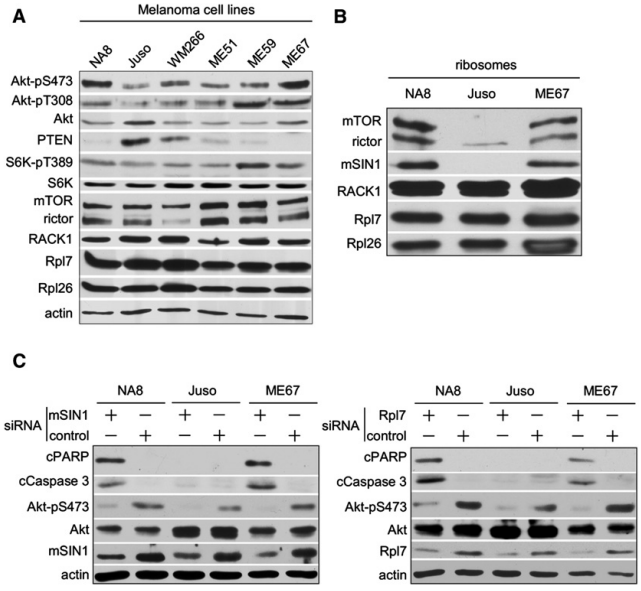

Figure 7. mTORC2-Ribosome Interaction Promotes Akt Signaling in Cancer Cells

(A) Akt Ser473 phosphorylation inversely correlates with PTEN expression in human melanoma cells. Cell extracts from six human melanoma cell lines were analyzed by immunoblotting with indicated antibodies.

(B) PTEN-deficient cell lines exhibit increased mTORC2-ribosome association. Ribosomes were purified from PTEN-deficient (NA8 and ME67) and PTEN-positive melanoma cell lines (Juso) by sedimentation through a sucrose cushion. Ribosomes were probed with the indicated antibodies.

(C) Knockdown of mSIN1 or Rpl7 induces apoptosis in PTEN-deficient (NA8 and ME67) melanoma cell lines. Cells were transfected with the indicated siRNA and harvested after 72 hr. Extracts were analyzed by immunoblotting to check the efficiency of the knockdown and the induction of apoptosis (PARP and caspase 3 cleavage) with the indicated antibodies.

See also Figure S7.

six human metastatic melanoma cell lines generated from different patients (Certa et al., 2001; Deschodt-Lanckman et al., 1990; Gervois et al., 1996). PTEN expression inversely correlated with Akt Ser473 phosphorylation (Figure 7A). For further study, we chose the two cell lines NA8 and ME67 with low PTEN expression and high Akt Ser473 phosphorylation (mTORC2 activity) and, as a control, the cell line Juso with the opposite signaling profile. To examine mTORC2-ribosome association, ribosomes were isolated from the three cell lines and probed for mTOR, rictor, and mSIN1. In all cases, the total yield of ribosomes obtained by sedimentation through a sucrose cushion was similar. However, the PTEN-deficient NA8 and ME67 cells exhibited significantly increased levels of mTORC2 associated with the ribosomal fraction as compared to the PTEN-positive Juso cells (Figure 7B), consistent with our PTEN knockdown studies in HeLa cells described above (Figure 6C). We also examined mTORC2-ribosome interaction in colon cancer cells harboring an activating mutation in the PI3K gene *PIK3CA*. The *PIK3CA* mutant cell lines HT29 and HCT116 exhibited both Akt Ser473 hyperphosphorylation (mTORC2 activity) and increased mTORC2-ribosome association as compared to SW60 colon cells harboring an unaltered *PIK3CA* gene (Figure S7A). Thus, mTORC2-ribosome interaction correlates with mTORC2 activity in both melanoma and colon cancer cells.

To investigate further the physiological relevance of mTORC2 activation via ribosome association, we examined the effect of mSIN1 and Rpl7 knockdown on mTORC2 signaling and cell survival in NA8, ME67, and Juso cells. Knockdown of either mSIN1 or Rpl7 decreased Akt Ser473 phosphorylation (Figure 7C), supporting a role for ribosome-dependent activation of mTORC2 in cancer. Furthermore, knockdown of either mSIN1 or Rpl7 enhanced apoptosis both in the presence and absence of etoposide. Interestingly, the increase in apoptosis was more pronounced in the PTEN-deficient NA8 and ME67 cells than in Juso cells, suggesting that NA8 and ME67 cells are more "addicted" to mTORC2 (Figure 7C and Figure S7B). The above results taken together suggest that mTORC2-ribosome association, mediating PI3K-mTORC2-Akt signaling and cell survival, is functionally important in cancer cells.

DISCUSSION

We investigated the upstream regulation of TORC2. A genetic screen in yeast and subsequent studies in mammalian cells revealed that ribosomes are upstream of TORC2. In particular, we found the following. First, the genetic screen in yeast revealed that knocking down ribosome biogenesis inhibits TORC2 kinase activity in vitro (Figure 1D) and TORC2 signaling in vivo (Figures 1B and 1C and Figure S1). Second, the ribosome is required for mTORC2 activity in vitro (Figure 2B and Figure 3B) and mTORC2 signaling in vivo in mammalian cells (Figure 2A, Figure 3A, and Figure S3A). Knockdown of ribosome maturation factor mNIP7 or ribosomal proteins (Rpl7 or Rps16) in mammalian cells decreased mTORC2 kinase activity and mTORC2 signaling. Third, the ribosome (translating or nontranslating) interacts directly with mTORC2 (Figures 5 and Figure S5). mTORC2 copurified with ribosomes isolated by four independent methods. Furthermore, mTORC2 copurified with ribosomes isolated from growing cells or cells treated with protein synthesis inhibitors. Fourth, ribosome-bound mTORC2 is active (Figure 5C), and ribosome-free mTORC2 is inactive in vitro (Figure 2B, Figure 3B, and Figure S6B). Fifth, PI3K-dependent insulin signaling stimulates binding of the ribosome to mTORC2 (Figure 6 and Figure S6D) with the same kinetics that it stimulates mTORC2

activation (Figure 6A). Finally, the mTORC2-ribosome interaction correlates with mTORC2 activity in both melanoma and colon cancer cells (Figure 7 and Figure S7). Melanoma and colon cancer cells with high PI3K activity (due to loss of PTEN or an activating mutation in the PI3K gene) exhibited both enhanced mTORC2-ribosome interaction and increased mTORC2 activity. Our findings suggest that the translating or nontranslating 80S ribosome binds and activates mTORC2 in response to growth factor-stimulated PI3K signaling (Figure S7C). TORC2-ribosome association is a mechanism of TORC2 activation that is likely conserved from unicellular yeast to human. The ribosome is presumably a primordial activator of TORC2 onto which growth factor signaling was grafted during the evolution of multicellularity.

In a parallel and complementary study, Oh et al. also showed that mTORC2 associates with the ribosome (Oh et al., 2010). Furthermore, they showed that mTORC2 phosphorylates the Akt turn motif cotranslationally and the Akt hydrophobic motif posttranslationally. Thus, Oh et al. investigated the role of the mTORC2-ribosome interaction in downstream signaling by mTORC2. Our study addresses the separate issue of upstream regulation of mTORC2. We show that an mTORC2-ribosome interaction activates mTORC2, and this activation is independent of translation. In other words, an mTORC2-ribosome interaction activates mTORC2 regardless of whether mTORC2 is phosphorylating a substrate co- or posttranslationally. We note that Oh et al. did not examine a requirement for the ribosome in posttranslational phosphorylation.

A connection between ribosomes and TOR signaling is well established. TORC1 activates ribosome biogenesis and protein synthesis and inhibits autophagy as key readouts in the control of cell growth. Why should ribosomes control TORC2? Ribosome content determines growth capacity of the cell and TORC2 regulates growth-related processes. Thus, regulation of TORC2 by ribosomes ensures that TORC2 is not inappropriately activated in cells that are unable to grow. The above also implies that TORC1, via activation of ribosome biogenesis and inhibition of autophagy-mediated ribosome turnover, indirectly controls TORC2. Indeed, Sarbassov et al. (Sarbassov et al., 2006) have shown that inhibition of mTORC1 by long-term rapamycin treatment indirectly inhibits mTORC2. Our findings suggest that the effect of rapamycin on mTORC2 is due, at least in part, to a reduction in ribosome content. Interestingly, the literature also indicates that ribosomal defects induce apoptotic cell death, although the underlying mechanism is not understood (Warner and McIntosh, 2009). We find that ribosomal defects inhibit mTORC2 and its downstream effector Akt, which in turn leads to Bad-dependent apoptosis. Thus, our findings also provide a mechanism for the induction of apoptosis by a ribosomal defect.

Our findings suggest that ribosomes bind and activate mTORC2 directly. The fraction of total mTORC2 that associates with ribosomes varies depending on the cell type and the growth conditions. For example, under normal growth conditions, ~20% of total rictor (mTORC2) was associated with ribosomes in HeLa cells, whereas ~30%–40% of rictor was associated with ribosomes in PTEN-deficient cells such as melanoma and PTEN knockdown cells. Thus, ribosome association appears to be a major if not the sole mechanism of TORC2 activation. Consideration of the fraction of total ribosomes that associate with mTORC2 is also potentially informative. Given that ribosomes are 100- to 1000-fold more abundant than signaling kinases such as mTORC2, only a small fraction of total ribosomes bind mTORC2. This excess of ribosomes would require strong regulation of mTORC2-ribosome binding by upstream PI3K signaling to achieve physiologically relevant regulation of mTORC2. Alternatively, TORC2 could be regulated by a specific subpopulation of ribosomes. Previous studies have demonstrated that TORC2 is associated with membranes, including the endoplasmic reticulum (ER) and Golgi apparatus, and that mTORC2 isolated from ER microsomes phosphorylates Akt Ser473 in vitro (Drenan et al., 2004; Hresko and Mueckler, 2005; Liu and Zheng, 2007; Schroder et al., 2007; Sturgill et al., 2008). These findings suggest that TORC2 might associate specifically with membrane-bound ribosomes. In support of this notion, we observed that mTORC2 copurifies with ribosomes only when the ribosomes are isolated in the presence of detergent. Membrane-bound ribosomes constitute ~10% of total ribosomes and include both translating and nontranslating ribosomes (Seiser and Nicchitta, 2000). It is also interesting to note that Komili et al. (Komili et al., 2007) have proposed a ribosome code in which there is a specialization of ribosomes for specific cellular process.

Our findings are consistent with other studies proposing the ribosome as a kinase platform. The two kinases PKCβII and Pim-1 are associated with the ribosome via RACK1 and Rps19, respectively (Ceci et al., 2003; Chiocchetti et al., 2005; Grosso et al., 2008b). Overall, the ribosome appears to be a signaling platform for mTORC2 and other kinases. Furthermore, ribosomal proteins have been shown to modulate the activity of NF-κB, p53, and c-Myc (Lindström, 2009).

PI3K-Akt signaling is upregulated and contributes to tumorigenesis in ~60% of advanced-stage melanomas (Stahl et al., 2003, 2004). PTEN expression or Akt inhibition increases sensitivity of melanoma cells to apoptosis-inducing agents and prevents tumor development (Madhunapantula and Robertson, 2009; Stahl et al., 2004). Our data, with melanoma, cancer colon, and HeLa cells, suggest that PI3K signaling promotes Akt phosphorylation via stimulation of mTORC2-ribosome binding. Furthermore, disruption of the mTORC2-ribosome supercomplex selectively induces apoptosis in PTEN-deficient melanoma cells (Figure 7C). The extent to which cells of other cancers require ribosome-dependent mTORC2 activation is unclear, although we expect that other cancers driven by mutations promoting PI3K signaling may also depend on mTORC2-ribosome association. Disrupting the mTORC2-ribosome interaction may be a useful strategy in the treatment of melanomas, colon carcinomas, and possibly other cancers.

Several findings suggest that upregulation of the protein synthesis machinery contributes to the development of cancer and other diseases (Ruggero and Pandolfi, 2003). Consistent with our findings, a ribosomal protein deficiency inhibits Akt-driven tumorigenesis (Hsieh et al., 2010). Furthermore, the *myc* oncogene enhances ribosome biogenesis, and *myc* oncogenicity in mice can be blocked by mutations in ribosomal protein genes (Barna et al., 2008; Ruggero, 2009). Our findings and the observation that mTORC2 is required for tumor progression in

at least some cancers (Guertin et al., 2009; Gulhati et al., 2009; Masri et al., 2007) suggest that myc and increased ribosomal content may promote tumorigenicity via stimulation of mTORC2 and its downstream effector Akt.

Curiously, our genetic screen in yeast yielded 44 TORC2 mutants but only a single mutant (*nip7-1*) that was defective in ribosome biogenesis. Why did we not obtain more mutants that were defective in ribosomal maturation factors or ribosomal proteins? First, ribosomal genes are duplicated in yeast, thereby precluding identification of recessive, loss-of-function mutations in these genes. Second, YPK2 is downstream of the ribosome in activation of TORC2 but is not downstream of the ribosome in mediating protein synthesis, precluding full suppression of a ribosome biogenesis defect by YPK2*. YPK2* only partly suppresses the *nip7-1* mutation and only at semipermissive temperature.

EXPERIMENTAL PROCEDURES

Detailed protocols for apoptosis assays and statistical analyses can be found in the Extended Experimental Procedures.

Yeast Strains, Media, Kinase Assays, Actin Staining, and Antibodies

Yeast strains used in this study are listed in the Table S1. All strains are isogenic derivatives of JK9-3d**a**. Plasmids used in this study are described in Table S2. Standard techniques and media were used for yeast manipulation (Kamada et al., 2005; Loewith et al., 2002). Unless indicated otherwise, cells were grown in rich YPD medium. YPK2 and TORC2 kinase assays were performed as described previously (Casamayor et al., 1999; Kamada et al., 2005; Loewith et al., 2002; Wullschleger et al., 2005). Rhodamine phalloidin staining of polymerized actin was performed as described (Loewith et al., 2002; Mulet et al., 2006). Immunoprecipitations were performed as described previously (Loewith et al., 2002; Wullschleger et al., 2005).

Reverse Suppressor Screen

A wild-type strain (JK9-3d**a**) was transformed with an *URA3*-based plasmid overexpressing YPK2* (pYPK2*) (Figure 1A). Cells were randomly mutagenized with 100 mM ethyl-methanesulfonate (EMS) (Sigma) for 15 min in SD-Ura medium, washed, and then allowed to recover in SD-Ura medium without mutagen for 2–4 hr. Cells were plated on solid SD-Ura or SD supplemented with 5-FOA and incubated at 30°C. The SD medium supplemented with 5-FOA counterselected against *URA3* such that only those cells that had spontaneously lost the *URA3*-based pYPK2* plasmid were able to form a colony. Mutants that were unable to grow on SD 5-FOA (and hence in the absence of YPK2*) were isolated from the master SD-Ura plate. ~45,000 colonies from mutagenized cells were screened, from which 45 mutants were isolated and the corresponding mutations were identified. Mutated genes were isolated by complementation with a *LEU2* centromeric plasmid-based yeast genomic library. Complementing members of the genomic library were selected by growth on SD-Leu medium containing 5-FOA. Genomic inserts of library-derived plasmids were identified by sequencing. Complementation with subclones of isolated inserts identified the complementing ORF within a given insert. Sequencing of the genomic copy of *NIP7* and meiotic segregation studies confirmed that *NIP7* was indeed the relevant mutant gene. *nip7-1* was found to be temperature sensitive upon subsequent characterization. For experiments at semipermissive temperature (30°C), *nip7-1* cells were grown in YPD at permissive temperature (25°C), diluted to OD_{600} = 0.1, and grown at 30°C to approximately OD_{600} = 0.6–0.8. For experiments at the nonpermissive temperature (37°C), *nip7-1* mutant was grown in YPD at 25°C and then shifted to 37°C for 6 hr.

Cell Culture, Immunoprecipitations, Immunoblotting, and mTORC2 Kinase Assay

HeLa, melanoma cells, and colon cancer cells were cultured, transfected, stimulated, and harvested as described previously (Jacinto et al., 2004; Thedieck et al., 2007). In brief, cells were seeded and grown for 48 hr in DMEM supplemented with 10% serum (basal conditions). Cells were starved of serum for 3 hr before restimulation with 100 nM insulin (Sigma).

For mSIN1, mNIP7, Rpl7, Rps16, PTEN, or Bad knockdown, a pool of four different synthetic siRNA or of the appropriate control siRNA (Dharmacon) was used as described (Thedieck et al., 2007). All transfections were done according to the manufacturer's instructions (Lipofectamine, Invitrogen transfection).

Protein extracts were prepared as previously described (Jacinto et al., 2004; Thedieck et al., 2007), resolved on SDS-PAGE, and transferred to nitrocellulose membranes (Protran, Whatman). Immunoprecipitation, immunoblotting, and mTORC2 kinase assays were performed as previously described (Jacinto et al., 2004; Thedieck et al., 2007).

Polysome Profiles, Ribosome Purification, and Poly(A) mRNA Pull-Down

Polysome analysis using sucrose gradients was performed as described previously (Grosso et al., 2008b; Idol et al., 2007). For ribosome purification by sedimentation through a sucrose cushion, HeLa or melanoma cells were washed in PBS, trypsinized, and lysed in buffer A (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 30 mM $MgCl_2$, 0.3% CHAPS, 100 ug/ml cycloheximide, 40 U/ml RNase inhibitor, protease inhibitor cocktail, and 100 ug/ml cycloheximide). Whole-cell extracts were clarified at 4°C, 10 min at 15,000 × g. Extracts were loaded on a 30% sucrose cushion in 50 mM Tris-acetate (pH 7.5), 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM DTT and ultracentrifuged for 17 hr in a SW41Ti Beckman rotor at 39,000 rpm. For the mTORC2 kinase assay, the ribosomal pellet was resuspended in mTORC2 kinase buffer (Jacinto et al., 2004; Thedieck et al., 2007). Ribosome-sucrose gradient fractionation was performed as previously described (Grosso et al., 2008a). For poly(A) pull-down, HeLa cells were washed with PBS, trypsinized, and lysed in buffer A. Whole-cell extracts were clarified at 4°C, 10 min at 8000 × g. Lysates corresponding to 5 × 10^7 cells were incubated with oligo(dT) cellulose (Invitrogen) for 1 hr at room temperature. After incubation, the oligo(dT) cellulose was pelleted and washed five times with buffer A. The bound fraction was eluted with elution buffer (100 mM Tris [pH 7.4], 500 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 5 mM DTT). Purified ribosome fractions and the bound and unbound fractions after poly(A) pull-down were concentrated with Vivaspin 500 (Sartorius Stedim) and analyzed by immunoblotting with the indicated antibodies.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2011.02.014.

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WORK EXPERIENCE

January 2015 – Present

Healthcare Consultant by Executive Insight

- Focus Area: Market Access, Customer & Market Insights
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November 2008 – July 2014

Researcher (PhD and 9 months Postdoctoral Fellow)
University of Basel, Biozentrum: Laboratory of Professor Michael N Hall

- Fields of research: broad range of molecular biology, yeast genetics and biochemistry approaches to understanding cell growth regulatory mechanisms, essential processes deregulated in tumors, with a particular focus on TOR (target of rapamycin) pathway.
- Experienced with mammalian and budding yeast tissue cultures; cloning; protein expression and purification; subcellular fractionation; ribosome profiling; kinase assay.
- Extensive computer experience with: Microsoft Office tools; Adobe Illustrator and Photoshop; Graphpad Prism; Bioinformatic tools.
- Teamwork skills developed through successful scientific collaborations leading to three publications.
- Project management: completed Basic Project Management Training (SPOL) in January 2014.
- Participated with oral and written presentations at various scientific conferences.
- Mentor and lecturer for undergraduate students.
- Theses' Supervisor: Bachelor (2007) and Master (2011-2012).
- Member of the PhD Students' Representatives: Biozentrum PhD Students. Representative (2011-2012) with specific responsibilities as Treasurer and Yearly PhD Student Retreat Organizer.
- Transferable skills: logical & innovative entrepreneurial attitudes; quick to grasp new concepts; hypothesis driven problem solving; data analysis, evaluation & synthesis; planning & organization; attention to relevant detail; collaborative teamwork & leadership; communication and internal & external presentation skills; mentoring; ability to work under pressure; comfortable with ambiguity.

May 2008 – September 2008 **Research Fellow (Postgraduate)**
University of Milano, Bicocca: Laboratory of Professor Massimo Labra

- Project Title: "Development of DNA molecular markers for varietal characterization and traceability of food".
- Field of research: plant biology, DNA barcoding.
- Developed expertise in following areas: DNA extraction, amplification & sequencing; bioinformatics tools for DNA analysis, comparison & phylogenesis.

EDUCATION

November 2008 – October 2013 **Philosophiæ Doctor in Biochemistry**
University of Basel, Biozentrum: Laboratory of Professor Michael N Hall
Thesis Title: "Upstream regulation of yeast TOR complexes".

January 2006 – April 2008 **Master of Science in Industrial Biotechnology, major Pharmacogenomics**
University of Milano, Bicocca: Laboratory of Professor Marina Vai
Thesis Title: "Gene expression and histone code in nitrogen starvation in *Saccharomyces cerevisiae*".

September 2001 – December 2005 **Bachelor of Science in Biotechnology, major Molecular Biotechnology**
University of Milano, Bicocca: Laboratory of Professor Maurizio Casiraghi
Thesis Title: "Bioinformatic tools applied to the analysis of the secretion system's proteins of *wolbachia*, symbiont of the filarial nematode *Dirofilaria immitis*".

OTHER ACHIEVEMENTS

Presentations and Publications

- Oral presentation at the Swiss Yeast Meeting (SYM) 2010.
- Poster presentations at the Biozentrum Symposium 2013, and Biozentrum PhD Students' Retreat 2009-2012.
- **Stracka D**, Jozefczuk S, Rudroff F, Sauer U, Hall MN. Nitrogen source activates TOR (Target Of Rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins. **J Biol Chem**. 2014 Sep 5;289(36):25010-20.
- Betz C, **Stracka D**, Prescianotto-Baschong C, Frieden M, Demaurex N, Hall MN. mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. **Proc Natl Acad Sci U S A**. 2013 Jul 30;110(31):12526-34.
- Zinzalla V, **Stracka D**, Oppliger W, Hall MN. Activation of mTORC2 by association with the ribosome. **Cell**. 2011 Mar 4;144(5):757-68.

Awards and Honors

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Hobbies and Passions

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