Control of ribosome biogenesis by the TORC1-Sch9 pathway in Saccharomyces cerevisiae

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Abstract

Yeast TORC1 is a evolutionarily conserved multiprotein complex whose kinase activity is ensured by the TOR catalytic subunit. In response to environmental cues, TORC1 directly phosphorylates and activates the AGC kinase Sch9 to regulate anabolic processes such as translation. We show here that Sch9 mediates TORC1 signals to all three nuclear RNA polymerases (RNAP I-III) via transcriptional repressors. Maf1 is directly phosphorylated and inhibited by Sch9 to prevent it from repressing RNAP III. Stb3, Dot6 and Tod6 are also directly phosphorylated by Sch9 to keep them from recruiting RPD3L upstream of ribosome biogenesis and ribosomal protein genes and thereby from repressing their transcription by RNAP II. In turn, expression of those genes serves to promote RNAP I activity.

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Résumé en français

Le complexe TOR 1 (TORC1) est un régulateur de croissance cellulaire conservé à travers l’évolution chez les eucaryotes permettant de contrôler les mécanismes de croissance intracellulaire en fonction des conditions environnementales. Il est aujourd’hui clairement établi que l’activité kinase de ce complexe est nécessaire non seulement pour stimuler les processus anaboliques mais aussi pour réprimer les processus cataboliques et l’induction de programmes transcriptionnels de réponse au stress incompatibles avec la croissance. Bien que ces nombreux effets distants de TORC1 soient connus grâce à son inhibiteur, la rapamycine, il est vraisemblable que d’autres effets restent encore à identifier. Bien des mécanismes moléculaires couplant TORC1 à ses effets distants connus ne sont également pas clairement éclaircis.

Pour répondre à ces questions fondamentales, d’une manière aussi compréhensive et non-biaisée que possible, nous avons utilisé une nouvelle technique de spectrométrie de masse sans marquage isotopique pour quantifier les changements de phosphorylation des protéines de *Saccharomyces cerevisiae* pendant un traitement avec la rapamycine. Ces cribles ont été répétés dans des mutants de *TAP42* et de la Ser/Thr kinase *SCH9* de la famille AGC, deux effecteurs directs de TORC1, pour déterminer si les phosphoprotéines identifiées sont régulées de manière dépendante à l’une ou l’autre de ces protéines. Les données générées par ces études ont permis l’identification de plus de 100 phosphorylations sensibles à la rapamycine, révélant ainsi de nombreux nouveaux aspects de la voie de signalisation de TORC1. Nous présentons dans ce travail nos efforts pour éclaircir de manière plus détaillée comment TORC1 stimule la biogénèse des ribosomes.

La biogénèse des ribosomes, qui est très strictement régulée par TORC1, ainsi que cela a été montré précédemment, est probablement le plus gros consommateur énergétique parmi tous les processus anaboliques de la cellule, en cela qu’il requiert l’activité transcriptionnelle des trois ARN polymérasases (RNA Pol) nucléaires et la traduction des très abondantes protéines ribosomale. Dans la continuation des résultats de nos cribles phosphoprotéomiques, nous montrons que Sch9 stimule
l’activité transcriptionnelle des RNA Pol I, II et III via la phosphorylation directe et l’inhibition de différents répresseurs de la transcription. Sch9 phosphoryle Maf1 afin de l’empêcher de se lier à et d’inhiber RNA Pol III, par là stimulant finalement la synthèse de l’ARN ribosomal 5S et des tRNAs, les essentiels cofacteurs des ribosomes. En plus de Maf1, Sch9 phosphoryle directement Stb3, Dot6 et Tod6 in vivo et in vitro. Nous montrons que ces facteurs, récemment impliqués dans la répression des gènes nécessaires à la biogénèse des ribosomes (ribi) et des gènes codant pour les protéines ribosomales (RP), servent à recruter le complexe déacétylase d’histones RPD3L aux promoteurs de leurs gènes cibles. La régulation des gènes ribi et RP influence à son tour la transcription du précurseur de l’ARN ribosomal 35S par RNA Pol I ainsi que sa maturation en ARN ribosomaux 25S, 18S, 5.8S.

Globalement, notre travail suggère que le réseau de signalisation de TORC1 est beaucoup plus étendu qu’anticipé précédemment et nous fournit des informations sur les mécanismes par lesquels TORC1 et Sch9 régule la biogénèse des ribosomes et, plus généralement, la croissance de S. cerevisiae.
Abstract

The Target Of Rapamycin Complex 1 (TORC1) is a conserved regulator of eukaryote growth, serving to couple environmental cues to the intracellular growth machinery. It is now clear that the Ser/Thr kinase activity of this complex is necessary not only to stimulate anabolic processes but also to repress catabolic processes and the transcriptional induction of stress response programs that are not compatible with growth. Although many distal readouts of TORC1 are known thanks to its small molecule inhibitor, rapamycin, more of them very likely remain to be identified. Also, how TORC1 regulates its known readouts is in many cases not well explained at the molecular level.

To address these fundamental questions in an as large-scale and unbiased manner as possible, we have used a novel, label-free mass spectroscopy approach to quantitate rapamycin-induced changes in protein phosphorylation in *Saccharomyces cerevisiae*. We repeated these screens in mutants of *TAP42* and the AGC family Ser/Thr kinase *SCH9*, two direct effectors of TORC1, to sort the regulated phosphoproteins according to their dependency on either of these two major TORC1 effectors. The data generated from these studies identified more than 100 rapamycin-sensitive phosphorylation events and revealed many new aspects of TORC1 signaling; here we present our efforts to probe in more detail how TORC1 promotes ribosome biogenesis.

Ribosome biogenesis, which had previously been shown to be under tight regulation by TORC1, is probably the most energy-consuming anabolic process of the cell as it requires transcription by all three nuclear RNA polymerases and translation of the very abundant ribosomal proteins. Building on results obtained in our phosphoproteomic dataset, we show that Sch9 stimulates RNA Pol I-, II- and III-dependent transcription via direct phosphorylation/inhibition of various transcription repressors. Sch9 phosphorylates Maf1 to prevent it from binding and repressing RNA Pol III, thereby ultimately promoting the synthesis of the 5S ribosomal RNA and the essential ribosomal cofactors, the tRNAs. In parallel to Maf1, Sch9 directly phosphorylates Stb3, Dot6 and Tod6 *in vivo* and *in vitro*. We showed that these recently identified repressors of genes involved in promoting ribosome biogenesis (ribi)
and ribosomal protein (RP) genes function by recruiting the RPD3L histone deacetylase complex to the promoters of their target genes. The regulation of ribi and RP genes in turn influences the transcription of the 35S precursor ribosomal RNA by RNA Pol I and its processing yielding the longer 25S, 18S and 5.8S ribosomal RNAs.

Altogether, our work suggests that the TORC1 signaling network is much vaster than previously anticipated and provides insights into the mechanisms by which TORC1 and Sch9 regulate ribosome biogenesis and, more generally, growth in S. cerevisiae.
I. Introduction

Rapamycin, a small molecule drug also known as sirolimus, and its derivatives are used today in the treatment of various diseases and conditions. Rapamycin was first used as an immunosuppressant in transplant patients (Calne et al., 1989) and is now used in stents to help prevent restenosis (Fattori and Piva, 2003). Rapamycin’s potent cytostatic effects against tumor cells prompted the development of derivatives with improved pharmacological properties. Two of those derivatives, temsirolimus and everolimus, are now used in the clinic to treat renal cell carcinomas and are being evaluated against other cancer types (Huber et al., 2009b).

Isolation of rapamycin as an antifungal

Rapamycin was first purified and isolated as the active principle produced by the bacterium Streptomyces hygroscopicus that killed the fungal pathogen Candida albicans (Vezina et al., 1975). This initial study revealed that it was extremely effective as an antifungal agent in the low nanomolar range (Sehgal et al., 1975). Structural analysis of the purified compound revealed an uncharged macrocyclic lactone synthesized in vivo by modification of a polyketide backbone Figure 1A). Beyond its antifungal properties, rapamycin was shown to function more generally as a cytostatic agent in eukaryotic cells ranging from yeast to animal cells, causing cells to arrest in the G1 phase of the cell cycle (Heitman et al., 1991).

Figure 1.  
A. Developed molecular structure of rapamycin. B. Developed molecular structure of FK506.  
C. Developed molecular structure of cyclosporin A. (Source: http://www.wikipedia.org/)
Macrocyclic immunosuppressants and their peptidyl-prolyl isomerases receptors

Two years after its discovery, rapamycin was described as an immunosuppressant in rats (Martel et al., 1977). This finding and the structural or mechanistic similarities of the molecule with FK506 and cyclosporin A, other macrocyclic immunosuppressants, ultimately led to the discovery of rapamycin's receptor, FKBP12. Like rapamycin, FK506 belongs to the macrocyclic lactone family (Figure 1B), while cyclosporin A is a structurally unrelated macrocyclic peptide (Figure 1C). The later compounds are also produced by soil microbes, Streptomyces tsukubaensis and Trichoderma polysporum respectively (Kino et al., 1987; Ruegger et al., 1976).

In addition to sharing a macrocyclic structure, the three immunosuppressants also share a common type of protein receptor (Table 1). Cyclosporin A was the first compound for which a receptor was discovered. This receptor was named cyclophilin for its biochemical affinity for the drug (Handschumacher et al., 1984). Subsequently, FK506 was found to bind directly to FKBP12 (FK506 Binding Protein 12 kDa) in human and bovine cytosolic extracts (Harding et al., 1989). Cyclophilin and FKBP12 are similar small cytosolic proteins with peptidyl-prolyl isomerase (PPIase) activity (Handschumacher et al., 1984; Harding et al., 1989).

Cyclosporin A and FK506 show virtually indistinguishable effects on the immune system, which suggested a common target and it was proposed that, despite their different receptors, the drug would then tether the PPIases to the same protein (Harding et al., 1989). The exact nature of the inhibition occasioned by this tethering mechanism is still unknown, but FK506 and cyclosporin A were indeed found to induce the formation of a complex between their respective receptor PPIases and a common target, the calcineurin phosphatase (Table 1) (Liu et al., 1991).
Table 1. Macrocyclic immunosuppressant and their molecular targets

<table>
<thead>
<tr>
<th>Macrocyclic compound</th>
<th>Receptor PPlase</th>
<th>Target protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>FKBP12</td>
<td>TOR1, TOR2</td>
<td>(Harding et al., 1989; Heitman et al., 1991; Koltin et al., 1991)</td>
</tr>
<tr>
<td>FK506</td>
<td>FKBP12</td>
<td>Calcineurin</td>
<td>(Harding et al., 1989; Liu et al., 1991)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>Cyclophilin</td>
<td>Calcineurin</td>
<td>(Handschumacher et al., 1984; Liu et al., 1991)</td>
</tr>
</tbody>
</table>

Rapamycin was first proposed to bind FKBP12 on the basis of its ability to compete with FK506 (Dumont et al., 1990; Harding et al., 1989). This hypothesis was later confirmed in genetic screens in *S. cerevisiae* where random mutations impairing the function of FKBP12 suppressed the cells’ sensitivity to rapamycin (Heitman et al., 1991; Koltin et al., 1991). Reverse genetic analysis confirmed that a complete deletion of the *FPR1* gene was viable (Heitman et al., 1991), showing none of the phenotypes observed upon exposure to rapamycin. This suggested that, similar to the situation with FK506 and cyclosporin A, FKBP12 was only functioning as a receptor for rapamycin not as its molecular target.

Identification of the Target Of Rapamycin genes

The vast majority of the spontaneous mutations identified in the genetic screens described above yielded mapped to the *FPR1* gene and yielded recessive resistance to rapamycin. However, on rare occasion, dominant mutations were found which mapped to two distinct complementation groups (Heitman et al., 1991). Subsequent to this initial genetic description, these two loci were cloned and found to encode highly similar genes which were named TOR1 and TOR2 (for Target Of Rapamycin) (Heitman et al., 1991). The mutants isolated had single missense mutations at a specific codon in a domain conserved in the two genes, probably explaining why they were less frequent compared to those in *FPR1* in which any loss of function causing mutation would yield rapamycin resistance. TOR genes appear to be conserved throughout all eukaryotic evolution. Interestingly, higher eukaryotes only have a single TOR kinase which was identified shortly after its yeast homologs; mTOR.
(mammalian Target Of Rapamycin) for example was identified via its affinity for FKBP12 in the presence of rapamycin (Brown et al., 1994; Sabatini et al., 1994).

**TOR proteins are kinases of the PIKK family**

The *TOR* genes encode kinases of the PIKK family (Phosphatidyl Inositol Kinase-like Kinase). PIKKs are huge kinases sharing a conserved domain structure most similar, as their name indicates, to Phosphatidyl Inositol 3 Kinases (PI3K; Figure 2). Despite their similarity to lipid kinases, all PIKKs, with the exception of TRRAP/Tra1 which has no kinase activity, were found to be Ser/Thr protein kinases (Keith and Schreiber, 1995; McMahon et al., 1998). Yeast Tor1 and Tor2 are composed of 2470 and 2474 amino acids respectively (~280 kDa), comprising all PIKKs common domains and phosphorylating proteins (Figure 2).

![Diagram of domain structure of yeast Tor1, mTOR and other selected PIKKs and PI3Ks](Lempiainen and Halazonetis, 2009).

Their catalytic domains are usually close to their C-termini, preceded by HEAT repeats (Huntingtin, Elongation factor 3, Alpha regulatory subunit of type 2A protein phosphatases and TOR1 repeats) (Perry and Kleckner, 2003). The repeats closest to the kinase domains form a FAT domain (FRAP-ATM-TRRAP domain) which is conserved at the sequence level among PIKKs (Bosotti et al.,...
At their very C-terminus, a FATC domain (FAT C-terminal domain) is also highly conserved, linked to the kinase domain by the less conserved PRD (PIKK Regulatory domain) (Bosotti et al., 2000; Mordes et al., 2008).

The mutations conferring dominant rapamycin resistance to the TOR proteins occurred in a conserved subdomain of the FAT domain, in close vicinity of the kinase domain. This sequence was found to be directly bound by rapamycin-FKB12 and is therefore referred to as the FRB (FKBP12-Rapamycin Binding) domain (Chen et al., 1995; Stan et al., 1994). The structure of the FKBP12-rapamycin-FRB complex revealed that rapamycin binds in hydrophobic clefts in both proteins and mediates most of the interactions between the two proteins. These observations explain the recruitment of FKBP12 to the TOR proteins only in the presence of rapamycin - the two proteins show virtually no affinity in the absence of the drug (Choi et al., 1996). The dominant rapamycin-resistant mutations in TOR occur at a specific position in the FRB domain and function by disrupting the FKBP12-rapamycin binding site.

The mechanism by which the FKBP12-rapamycin complex inhibits TOR kinases activity is still unclear. It was proposed early on that tethered FKBP12 would alter the conformation of proline residues in TOR proteins, but this hypothesis is unlikely since rapamycin and FK506 inhibit FKBP12 PPlase activity (Koltin et al., 1991). Other potential mechanisms could involve the trapping of a labile inactive conformation of the TOR kinases by the complex or steric hindrance of substrate recruitment. Interestingly, point mutations in the FRB domain were reported to hyperactivate the TOR kinase, underlining the importance of this domain for the regulation of the kinase (Ohne et al., 2008; Reinke et al., 2006).

No complete atomic resolution structure of any of the known PIKKs has ever been published to date. Of TOR kinases, only low resolution electron microscopy structures of entire PIKKs and crystal or NMR structures of small domains have yet been solved (Adami et al., 2007; Choi et al., 1996; Dames et al., 2005; Yip et al., 2010). An in silico model of the FAT, kinase and FATC domains of TOR
kinases has also recently been generated based on their similarity to PI3KCy whose structure has been solved by crystallography (Sturgill and Hall, 2009).

Although detailed structural information is still lacking, functional studies have shown that the FATC domain is essential for the activity of TOR kinases. Its complete or partial truncation results in loss of kinase activity (Takahashi et al., 2000); deletion of a single amino acid at the C-terminus of TOR proteins in the FATC domain can render the kinase inactive (Peterson et al., 2000).

The PR domain is less conserved within the PIKK family, but seems of importance for the regulation of kinase activity. Truncation of the N-terminal part of the PR domain in mTOR leads to the hyperactivation of the kinase, as does the binding of an antibody raised against this subdomain (Brunn et al., 1997; Sekulic et al., 2000).

**Target Of Rapamycin signaling in *Saccharomyces cerevisiae***

Yeasts have the particularity of bearing two paralogous TOR genes, while other eukaryotes usually only have one (De Virgilio and Loewith, 2006b). This particularity has been very useful to study TOR signaling thanks to various differences in phenotypes observed upon exposure to rapamycin or in mutants lacking one or both of the TOR genes.

**Overview of rapamycin effects in budding yeast**

As suggested above, budding yeast cells are sensitive to rapamycin which prevents their growth and ultimately causes their death (Heitman et al., 1991; Koltin et al., 1991). Like cells from many different species, yeast cells treated with rapamycin arrest in the G1 phase of the cell cycle (Heitman et al., 1991) and progressively activate stress/starvation transcriptional programs to ultimately show G0 cells traits (Barbet et al., 1996; Zaragoza et al., 1998). In some strain backgrounds this phenomenon can be reversed by washing out rapamycin from the medium (Dubouloz et al., 2005).

**TOR functions**

Reverse genetic analysis of the TOR genes in the budding yeast provided great insights into their functions. *TOR2* functions are essential for yeast cells survival while *TOR1* can be deleted causing a
significant growth phenotype (Kunz et al., 1993). Globally, tor1 mutant cells show phenotypes mimicking mild exposure to rapamycin: the cell cycle is not arrested but is delayed in G1 and cells accumulate glycogen and partially activate stress/starvation transcriptional programs. On the other hand, loss of TOR2 functions results in cells arresting randomly at all phases of their cycle. This reveals that TOR2 has essential functions which are not redundantly achieved by TOR1 (Helliwell et al., 1994; Kunz et al., 1993; Zheng et al., 1995). Loss of function of both TOR1 and TOR2 phenocopies treatment with rapamycin with cells arresting in G1 and showing pronounced G0 traits (Barbet et al., 1996). TOR1 and TOR2 therefore share common functions in cell cycle progression in G1 in parallel to functions specific to TOR2.

These results combined with the direct physical interaction between TOR proteins and the FKBP12-rapamycin complex and the fact that missense mutations in either TOR1 or TOR2 yields dominant rapamycin resistance strongly supports the notion that rapamycin interferes with the common function of both TOR genes but not the functions specific to TOR2.

**Two distinct TOR complexes**

*Biochemical identification of partners of Tor1 and Tor2*

The biochemical basis of those genetic observations was only elucidated with the purification of TOR proteins in complex with their various associated proteins (Loewith et al., 2002). In this study, epitope-tagged Tor1 and Tor2 proteins were immunopurified and associated proteins identified by mass spectrometry. These observations led to the discovery of two distinct multiprotein complexes: TOR complex 1 (TORC1) comprising either Tor1 or Tor2 associated with Lst8 and Kog1; and, TOR complex 2 (TORC2) composed of only Tor2 as a catalytic subunit bound by Lst8, Avo1, Avo2 and Avo3. Tco89 and Bit61/Bit2 were found in a later study to be part of TORC1 and TORC2 respectively (Reinke et al., 2004). In agreement with genetic data, the rapamycin-FKBP12 complex was found to interact with and thus inhibit only TORC1, which performs the common function of TOR1 and TOR2, while TORC2 was proposed to perform the rapamycin-insensitive functions of TOR2 (Loewith et al., 2002).
Complex | Yeast protein | Phenotype of yeast deletion | Mammalian homolog | References
---|---|---|---|---
TORC1 | Tor1 or Tor2 | Inviable<sup>a</sup> | mTOR | (Brown et al., 1994; Heitman et al., 1991; Loewith et al., 2002; Sabatini et al., 1994)
 | Lst8 | Inviable | mLST8 (GβL) | (Kim et al., 2003; Loewith et al., 2002)
 | Kog1 | Inviable | Raptor | (Hara et al., 2002; Kim et al., 2002a; Loewith et al., 2002)
 | Tco89 | Slow growth | - | (Reinke et al., 2004)
TORC2 | Tor2 | Inviable | mTOR | (Brown et al., 1994; Heitman et al., 1991; Loewith et al., 2002; Sabatini et al., 1994)
 | Lst8 | Inviable | mLST8 (GβL) | (Kim et al., 2003; Loewith et al., 2002)
 | Avo1 | Inviable | mSin1 | (Frias et al., 2006; Loewith et al., 2002)
 | Avo2 | viable | - | (Loewith et al., 2002)
 | Avo3 (Tsc11) | inviable | Rictor | (Loewith et al., 2002; Sarbassov et al., 2004)
 | Bit61 | viable | - | (Reinke et al., 2004)

<sup>a</sup> Loss of TOR1 function alone is not inviable.

Table 2. TOR complexes proteins in yeast and humans

**TORC1, a rapamycin-sensitive controller of growth**

**TORC1 is regulated by environmental cues**

TORC1 was first proposed to integrate environmental cues, and particularly reduced nitrogen source quality, based on the similarity of rapamycin-treated cells with nutrient-starved cells (De Virgilio and Loewith, 2006b). TORC1 activity in yeast was only recently confirmed to be stimulated by nutrients thanks to the discovery of its first *bona fide* physiological substrate, Sch9, whose phosphorylation can be monitored *in vivo* (Urban et al., 2007). TORC1 was in fact found to be downregulated in all conditions where growth should be paused or stopped; carbon and nitrogen starvation and a plethora of noxious stresses including oxidative stress, osmotic shock and heat shock all lead to TORC1 inhibition (Figure 3) (Urban et al., 2007).
Intracellular cues also modulate TORC1 activity

TORC1 was also found to sense the state of various cell processes such as ribosome biogenesis, translation, protein folding and vesicle trafficking. Impairment of ribosome biogenesis or translation was found to cause TORC1 hyperactivation (Lempiainen et al., 2009; Urban et al., 2007). As TORC1 stimulates both of those processes, these regulatory mechanisms may be considered as negative feedback loops serving to compensate their eventual defects (See Regulation of anabolic processes and Ribosome biogenesis homeostasis and negative feedback on TORC1). Conversely, inhibition of protein folding or vesicle trafficking leads to TORC1 downregulation (Devasahayam et al., 2006; Puria et al., 2008; Urban et al., 2007).

The Tap42-PP2A pathway

A major signaling branch downstream of TORC1 involves the type 2A and type 2A-related phosphatases and their essential regulatory protein Tap42. Tap42 was first identified in a screen for multicopy suppressors of temperature-sensitivity alleles of the aforementioned phosphatase genes.
and was then found to physically interact with these enzymes (Di Como and Arndt, 1996). This study also uncovered that a temperature-sensitive allele of TAP42, tap42-11, showed resistance to rapamycin at permissive temperature, suggesting a functional link to TORC1 signaling. It was later found that many temperature-sensitive alleles of TAP42 prevent many downstream signaling events normally elicited by rapamycin treatment and thereby enhance the cells resistance to rapamycin (Duvel et al., 2003). Rapamycin-resistant alleles of Tap42 are very useful tools to determine if readouts of TORC1 are regulated through the Tap42-PP2A pathway as mentioned in the following sections. Biochemical experiments revealed that Tap42 physically interacts with and is phosphorylated by TORC1 (Jiang and Broach, 1999). Furthermore the interaction of Tap42 with its partner phosphatases was shown to be sensitive to rapamycin (Jiang and Broach, 1999). Altogether these observations suggest that Tap42 serves as a direct target to mediate TORC1 signals to regulate the type 2A and type 2A-related phosphatases. It is however unclear whether all phosphatase-mediated signals from TORC1 are dependent on Tap42.

**Regulation of anabolic processes**

Abundant RNA and protein biosynthesis is a central aspect of growth that is regulated by TORC1. Ribosome biosynthesis is an energetically costly process for yeast cells and requires a substantial part of the cells’ synthesis capacity. Its regulation by TORC1 is detailed in a later section (*TORC1 regulates all aspects of ribosome biogenesis*). In addition to ribosome biogenesis regulation, TORC1 was found to be critical regulator of translation, impinging upon the initiation step (Barbet et al., 1996). The exact mechanisms by which TORC1 regulates that process are not fully elucidated but it involves, at least in part, Gcn2 (Figure 3). The Gcn2 kinase is a sensor activated by uncharged tRNAs to phosphorylate the α subunit of the eukaryotic Initiation Factor 2 (eIF2) to prevent its activation and function in translation initiation (Hinnebusch, 1993). TORC1 downregulates the sensitivity of Gcn2 to uncharged tRNAs and thereby controls its basal activity (Cherkasova and Hinnebusch, 2003; Garcia-Barrio et al., 2002). Gcn2 regulation by TORC1 was shown to occur by phosphorylation partially in Tap42-dependent manner (Cherkasova and Hinnebusch, 2003). TORC1 was additionally shown to
stabilize the translation initiation factor eIF4G (Berset et al., 1998). Importantly, the timeframe of translation initiation inhibition by rapamycin is fully compatible with mechanisms involving transcriptional regulation (Barbet et al., 1996). Interestingly, TORC1 is known to regulate genes involved in translation, such as TIF4631, encoding a copy of eIF4G, at the transcriptional level as part of the ribi regulon (see Ribosome biogenesis genes) (Wade et al., 2006) and could therefore also regulate translation by this mechanism.

**Repression of autophagy**

In parallel to stimulating anabolic processes, TORC1 inhibits catabolic processes such as autophagy (Figure 3). Autophagy, literally “self-eating”, is a recycling process involving the digestion of macromolecules and/or whole organelles to release building blocks necessary for de novo synthesis of proteins, RNAs and lipids (Diaz-Troya et al., 2008). Autophagy is minimal in exponentially growing yeast cells and is activated upon nitrogen/carbon starvation (Diaz-Troya et al., 2008). Like starvation, rapamycin treatment triggers activation of the Atg1 kinase complex, a central activator of autophagy, at least in part by causing Atg13, a direct TORC1 substrate, to become dephosphorylated and bind to the complex (Kamada, 2010; Kamada et al., 2000; Kamada et al., 2010).

**Repression of stress/starvation transcriptional responses**

In addition to the regulation of anabolic and catabolic processes, TORC1 represses various transcriptional programs which are incompatible with fast growth in good nutrient conditions. Namely, TORC1 represses the Nitrogen Discrimination Pathway (NDP) controlled by the Gln3 and Gat1 transcriptional activators, the Retrograde Pathway activated by Rtg1/3 transcription factors, the General Stress Response under the control of the Msn2/4 transcriptional factors and the G0 transcriptional program through its activator Gis1 (Figure 3) (Beck and Hall, 1999; Komeili et al., 2000; Pedruzzi et al., 2000; Pedruzzi et al., 2003). Consistently with the regulation of their target genes upon TORC1 inhibition, Gln3, Gat1, Rtg1/3 and Msn2/4 are kept out of the nucleus in optimal growth conditions and only concentrate there upon rapamycin treatment, exposure to noxious stress, starvation or changes in nutrient quality (Beck and Hall, 1999; Komeili et al., 2000). Regulation
of Gln3 and Gat1 by TORC1 depends on Tap42 (Beck and Hall, 1999). The last transcription factor, Gis1, is activated by the Rim15 kinase which is retained in the cytoplasm in a TORC1/Sch9-dependent manner (Pedruzzi et al., 2003; Urban et al., 2007; Wanke et al., 2008).

**TORC2 and the rapamycin-insensitive TOR functions**

Relative to TORC1, TORC2 functions are far less understood because of the lack of a convenient rapamycin-like tool. Reverse genetic analyses of TORC2 functions have mainly relied on tor2 temperature-sensitive mutants or dominant negative alleles of the TOR2 gene (Helliwell et al., 1998a; Helliwell et al., 1998b; Schmidt et al., 1996). Genetic screens with these tools lead to the observation that hyperactive versions of the Ypk1/2 kinases suppress the lethality of TOR2 loss of function (Kamada et al., 2005). In the same study, YPKs were shown to be directly phosphorylated at their C-terminus and thereby activated by TORC2 in a similar way as p70 S6 kinases by mTORC1 in mammals (Kamada et al., 2005). Ypks are considered to be the homologs of mammalian Serum- and Glucocorticoid-dependent Kinases (SGKs) which were later found to be themselves phosphorylated and activated by mTORC2 (Garcia-Martinez and Alessi, 2008; Yan et al., 2008).

Loss of TOR2 function causes defects in the cell cycle dependent polarization of the actin cytoskeleton polarization towards the bud, a process necessary for proper delivery of proteins and vesicles to the daughter cell (Helliwell et al., 1998b; Schmidt et al., 1996). This phenotype is suppressed by Ypk hyperactivation, suggesting that they mediate TORC2 signals to regulate actin polarization (Kamada et al., 2005).

**Sch9 signaling**

**Identification as a suppressor of PKA signaling deficiencies**

Sch9 is a Ser/Thr protein kinase of the AGC family. It was first identified as a multicopy suppressor of the lethal loss of function of CDC25 which encodes the RAS GTP Exchange Factor (RAS-GEF), an upstream activator of Protein Kinase A (PKA) (Toda et al., 1988). PKA activity is essential in yeast and loss of its three semi-redundant subunits, Tpk1, 2 and 3 can also be suppressed by Sch9
overexpression (Toda et al., 1988). Conversely, Sch9 disruption leads to strong growth impairment, a phenotype that may be partially suppressed by PKA hyperactivation (Toda et al., 1988). As these initial observations suggested, Sch9 was found later on to regulate various common cellular processes with PKA, which are described below.

Inhibition of the G0 transcriptional program and lifespan regulation

There are two measures of longevity commonly employed to measure lifespan in yeast. First, replicative lifespan measures the number of daughters generated by a mother cell before reaching senescence (Steinkraus et al., 2008). Second, chronological lifespan measures the time that yeast cells survive in stationary phase after consuming all nutrients in their direct environment (Paola and Valter, 2003). Artificial downregulation of PKA activity leads to a prolonged chronological lifespan which depends on the enhanced activity of Rim15 and its targets, the Msn2/4 transcription factors (Wei et al., 2008). Similarly, loss of Sch9 activity allows the cells to survive longer in stationary phase in a Rim15-dependent manner (Wei et al., 2008). This observation is corroborated by the fact that Sch9 was shown to directly phosphorylate Rim15 and, like TORC1, to prevent Rim15 accumulation in the nucleus (Pedruzzi et al., 2003; Wanke et al., 2008). Like PKA, Sch9 also functions to downregulate replicative lifespan, although the pathways connecting these kinases to this readout are not clearly identified yet (Kaeberlein et al., 2005).

Regulation of anabolic processes

Like TORC1 and PKA, Sch9 promotes ribi/RP gene transcription and translation initiation (Crauwels et al., 1997; Jorgensen et al., 2004). Sch9 appears to regulate these genes via the Stb3, Dot6 and Tod6 transcription repressors (see Stb3 binds to RRPE elements and Dot6 and Tod6 bind to PAC elements to repress ribi genes).

Repression of autophagy

Simultaneous inhibition of PKA and Sch9 leads to a mild activation of autophagy, while inhibition of either of the two kinases alone does not show any effect (Yorimitsu et al., 2007). Autophagy thus
triggered is dependent on the Atg1 kinase but, surprisingly, unlike autophagy induced upon TORC1 inhibition, does not involve Atg13 dephosphorylation (Yorimitsu et al., 2007). The Msn2/4 transcription factors and the Rim15 kinase were also shown to play a role but are dispensable for the initial activation of autophagy and only regulate the magnitude of the autophagic response (Yorimitsu et al., 2007).

**Phenotypic suppression of *sch9* cells**

Many studies of Sch9 functions rely on the usage of *sch9* strains. Although loss of function studies can normally be very informative, specific caveats in the case of *sch9* mutants likely distort some conclusions. *SCH9* loss of function leads to a strong growth phenotype in spores freshly dissected from heterozygous diploids. However, *sch9* cells promptly accumulate mutations and/or epigenetic alterations which suppress their growth impairment (Jorgensen et al., 2004). Although the exact nature of these alterations was never determined, they are likely to involve the hyperactivation of PKA (Toda et al., 1988) or the impaired function of negative regulators of growth downstream of Sch9. In either case these secondary effects will confound conclusions drawn solely on the basis of observations made in *sch9* cells.

**Ribosome biogenesis in *S. cerevisiae***

A highly coordinated process involving all three nuclear polymerases

The ribosome is the central enzyme of translation that reads mRNA templates to synthesize proteins. It catalyzes the formation of peptide bonds between amino acids with the help of its cofactors, the transfer RNAs (tRNAs) onto which the amino acids are loaded. The ribosome is composed of two subunits named after their sedimentation coefficients (60S for the large subunit and 40S for the small subunit in yeast). The subunits consist of an assembly of 78 ribosomal proteins with the 25S, 5.8S and 5S ribosomal RNA (rRNA) molecules for the 60S subunit or with the 18S rRNA for its 40S counterpart (Warner, 1999).
Ribosome biogenesis is the process encompassing synthesis, post-translational or post-transcriptional modification and assembly of the ribosomal components into ribosomes. Ribosome biogenesis is a very complex and highly coordinated process. All components need to be synthesized in approximately equimolar levels and must be modified and assembled in a timely fashion to ensure the production of functional ribosomes.

This central cellular process takes place in various locations in the cell. RP genes are transcribed in the nucleus, their mRNAs are translated in the cytoplasm and RPs are imported back into the nucleus (Warner, 1999). The transcription of rRNAs and the first processing and assembly steps take place in a crescent-shaped subcompartment of the nucleus referred to as the nucleolus (Venema and Tollervey, 1999). Later steps of ribosomal maturation then take place in the nucleus and in the cytoplasm after pre-40S and pre-60S subunits are exported through nucleopores (Kressler et al., 2010; Venema and Tollervey, 1999; Warner, 1999).

Ribosome biogenesis requires the concerted activity of the three yeast nuclear RNA polymerases (RNA Pol I-III). RNA Pol I is dedicated to the synthesis of the 35S pre-rRNA transcript which is later processed by various nucleases to yield the 25S, 18S and 5.8S rRNAs. RNA Pol II is necessary to transcribe the mRNAs of two distinct regulons: the ribi (ribosome biogenesis) and the RP (Ribosomal Protein) regulons. Last but not least, RNA Pol III transcribes the 5S rRNA, as well as tRNA cofactors.

**TORC1 regulates all aspects of ribosome biogenesis**

Ribosome biogenesis is energetically very costly for the growing cell. It is estimated that rRNA synthesis by odd RNA polymerases (i.e. RNA Pol I and III) accounts for 60% of total transcription while 50% of RNA Pol II activity is devoted to the expression of RP mRNA which then of course needs to be translated (Warner, 1999). Ribosome biogenesis therefore needs to be tightly regulated according to environmental conditions in order for the cells to cope with harsher times like nutrient deprivation.

It is perhaps not surprising that TORC1, which is regulated according to the presence or absence of nutrients and noxious stressors, plays a prominent role in regulating ribosome biogenesis. Given the highly coordinated nature of the process, it is maybe not surprising either that TORC1 was shown
to promote the activity of all three nuclear RNA polymerases to stimulate ribosome biogenesis (De Virgilio and Loewith, 2006a; Powers and Walter, 1999; Zaragoza et al., 1998). In the following sections, the mechanism of regulation of each of the three polymerases is reviewed with special emphasis on how TORC1 is proposed to impinge on their activity for proper control of ribosome biosynthesis.

**RNA polymerase I synthesizes the 25S, 18S and 5.8S ribosomal RNAs**

rDNA tandem repeats, chromatin structure and transcription rate

As mentioned above, RNA Pol I is in charge of transcribing the 35S rRNA precursor transcript which is later cleaved and modified to yield mature rRNAs of both ribosomal subunits (Venema and Tollervey, 1999). The 35S pre-rRNA gene is part of the ribosomal DNA (rDNA) tandem repeats on the right arm of chromosome XII in yeast (Figure 4A). Because of its repetitive nature and its consequent susceptibility to homologous recombination, the number of copies of the repeats varies from strain to strain with most strains harboring around 100-200 units (Warner, 1999). Analysis of the rDNA chromatin shows that only ~50% of rDNA repeats are actively transcribed by RNA Pol I even in exponentially growing cells in rich medium (Dammann et al., 1993). The number of rDNA repeats can be artificially reduced to less than 50 copies per cell without obvious consequences on growth as the cell can compensate by activating more genes and by transcribing them at a higher rate (French et al., 2003). The exact function of the large number of transcriptionally inactive copies is only beginning to be understood; one recent report showed a protective role against DNA damage indirectly caused by high transcriptional activity (Ide et al., 2010). Active rDNA repeats were shown to adopt an “open” chromatin state largely devoid of histones, which is set randomly across the array at each cell division (Dammann et al., 1993, 1995).
Figure 4. A. Scheme of rDNA tandem repeats. ETS: External Transcribed Spacer; ITS: Internal Transcribed Spacer. B. RNA Pol I transcription cycle. 1) RNA Pol I associates with Rrn3. 2) Rrn3-RNA Pol I then interacts with the CF complex and gets recruited to the 35S pre-rRNA promoter with the help of TBP and the UAF complex which binds to the upstream element in a stable manner. 3) RNA Pol I initiates transcription and Rrn3 dissociates from it. 4) RNA Pol I terminates transcription and is released from its DNA template. Known or potential (?) TORC1-regulated steps are indicated.

Entry into stationary phase and nutrient starvation both cause a drastic downregulation of 35S pre-rRNA transcription (Ju and Warner, 1994; Tsang et al., 2003). This effect is only very partially accounted for by a reduction in the number of active “open” rDNA repeats which only drops by 50% upon entry into stationary phase (Dammann et al., 1993). Disruption of the Rpd3 histone deacetylase (See Rpd3, a class I histone deacetylase) prevents “open” rDNA repeats to inactivate upon entry into stationary phase (Sandmeier et al., 2002). However global 35S pre-rRNA transcription is not derepressed in this mutant as the transcription rates of each individual active rDNA repeat is downregulated so as to compensate the higher number of active genes. On the other hand, RNA Pol I disruption causes the rDNA repeats to adopt a “closed” conformation (Dammann et al., 1995). Altogether these observations support a general model in which the global transcription rate potential of RNA Pol I is the predominant parameter in the regulation of 35S pre-rRNA synthesis rather than the number of active rDNA repeats (Ju and Warner, 1994; Oakes et al., 2006) and that,
besides other possible parallel regulatory mechanisms, RNA Pol I activity plays a role in the setting of appropriate rDNA chromatin conformation.

**TORC1 regulates RNA Pol I recruitment to the rDNA**

TORC1 has been proposed to promote RNA Pol I activity via several distinct mechanisms, but the stimulation of transcription rates rather than the alteration of the proportion of active rDNA repeats seems to account for most of the regulation (Claypool et al., 2004).

RNA Pol I transcription rates are mainly regulated at the transcription initiation stage (French et al., 2003). Several factors are required for proper initiation at the 35S pre-rRNA gene promoter (Figure 4B). The essential CF (Core Factor) complex interacts with the proximal core promoter element in a labile manner and is absolutely essential for cell viability (Keys et al., 1994; Lin et al., 1996; Nogi et al., 1991). Binding stably to a more distal element, the UAF (Upstream Activating Factor) complex is also necessary to ensure RNA Pol I transcription initiation by helping the recruitment of the CF and the TATA-binding protein (Keys et al., 1996; Nogi et al., 1991; Steffan et al., 1996).

RNA Pol I is recruited to the 35S pre-rRNA promoter by an additional factor, Rrn3, with which it forms a labile complex (Milkereit and Tschochner, 1998; Nogi et al., 1991; Yamamoto et al., 1996). Only a small fraction of RNA Pol I interacts with Rrn3 which then contacts the Rrn6 subunit of the CF complex to target the polymerase to the promoter (Peyroche et al., 2000). Shortly after transcription initiation, Rrn3 is inactivated and dissociates from RNA Pol I and both proteins need to associate *de novo* to complete the cycle (Hirschler-Laszkiewicz et al., 2003; Milkereit and Tschochner, 1998).

TORC1 was shown to promote Rrn3 association with RNA Pol I to enhance its recruitment to the rDNA (Claypool et al., 2004). Expression of a fusion construct mimicking constitutive interaction between Rrn3 and the Rpa43 subunit of the polymerase provides a bypass to the repression observed upon rapamycin treatment (Laferte et al., 2006). The exact mechanism by which TORC1 regulates the Rrn3-RNA Pol I interaction is not clear yet. However, the phosphorylation states of Rrn3 and RNA Pol I were shown to play a role in their interaction (Fath et al., 2001) and TORC1 could
possibly regulate these post-translational modifications either directly or via other kinases or phosphatases. It is also possible that Rrn3 is regulated by TORC1 at the expression level as it belongs to the *ribi* regulon (Wade et al., 2006), but such a mechanism would be independent of the control of Rrn3 affinity for RNA Pol I.

Another mechanism of regulation of RNA Pol I transcription initiation involves a sub-complex of the U3 snoRNP (small nucleolar RiboNucleoProtein) particle which functions in the processing of the primary 35S transcript in the 5’ ETS (Figure 4A) (Gallagher et al., 2004). The U3 snoRNP is composed of three subcomplexes, one of which, the t-UTP complex, associates with the rDNA chromatin in the 5’ external transcribed spacer just downstream of the RNA Pol I transcriptional start site in a transcription-independent manner. Artificial repression of any the t-UTP components leads to a strong downregulation of 35-pre-rRNA production. Although this mechanism has not been formally linked with TORC1, it is known that rapamycin treatment potently downregulates the expression of the subunits of the t-UTP complex as, like Rrn3, they are part of the *ribi* regulon (Wade et al., 2006).

TORC1 and the nucleolar structure

In parallel to the downregulation of RNA Pol I, rapamycin treatment is accompanied with massive changes in nucleolar structure and size. The nucleolus is usually a crescent-shaped compartment along the side of the inner nuclear envelope in rapidly growing cells. TORC1 inhibition results in a shrinkage of the nucleolus to a small round shaped structure which still contacts the nuclear envelope but through a much smaller surface (Tsang et al., 2003). Like the repression of rDNA repeats upon entry into stationary phase, this structural effect depends on the function of Rpd3 which is recruited to the rDNA chromatin upon rapamycin treatment (See *Rpd3, a class I histone deacetylase*). Rpd3 then deacetylates histone 4 lysines 5 and 12 (H4K5 and 12) at the rDNA and thereby promotes the loading of condensin which is necessary and sufficient to cause the nucleolus to shrink (Tsang et al., 2003; Tsang et al., 2007). Although it is known that TORC1 inhibition causes RNA Pol I to diffuse out of the nucleolus and that the presence of RNA Pol I at the rDNA participates in the interaction between the rDNA and the nuclear envelope, the Rpd3-mediated condensin
loading and nucleolar shrinkage processes appear to function in an independent manner (Oakes et al., 1998; Tsang et al., 2003; Tsang et al., 2007). It is not clear whether this pathway influences transcription by RNA Pol I, but the fact that Rpd3 deletion does not suppress RNA Pol I downregulation upon rapamycin treatment suggests that the two processes are parallel (Oakes et al., 2006).

**RNA Polymerase II and ribi/RP genes regulation**

In addition to RNA Pol I, TORC1 regulates many protein-coding genes transcribed by RNA Pol II. Among them are the ribi and RP genes whose expression is stimulated by TORC1. Although the expression of both regulons is regulated in the same direction by TORC1, the structure of their promoters and their timing of regulation differ. In specific mutants and/or conditions, their regulation may even be functionally uncoupled (Laferte et al., 2006). Many transcription factors have now been found to bind to the promoters of one or both of these sets of genes and/or to regulate their expression, but it is still not very well described how TORC1 signals are exactly transmitted.

**Ribosomal protein genes**

Ribosomal protein gene promoters have been intensively studied and are thought to contain at least four elements. Binding sites for the transcription factors Rap1 and Abf1 were the first to be identified. Rap1 binding motifs are found in most RP genes while Abf1 binding sites are only observed in a few cases, the two types of motifs being usually exclusive (Goncalves et al., 1995; Mager and Planta, 1990). An A/T-rich element, usually positioned downstream of the Rap1 and Abf1 binding sites, is also enriched in those promoters and is necessary to achieve full transcriptional activation (Goncalves et al., 1995; Mager and Planta, 1990). To our knowledge, no *trans*-acting factor has ever been formally described to bind that element. The last element is a binding site for the Fhl1/ifh1 transcription factors (Schawalder et al., 2004; Wade et al., 2004).

Rap1 is a multifunctional protein which independently serves to maintain telomere structure to silence transcription near telomeres and at the mating type loci and to activate the transcription of various genes including *RP* genes (Shore, 1994). Rap1 binding sites were shown to be essential for
the regulation of $RP$ genes according to nutrient availability (Goncalves et al., 1995). However, Rap1 binding in RP gene promoters was found to be largely independent of transcription regulation by TORC1 suggesting that its signals are mediated through other transcription factors (Rohde and Cardenas, 2003). Quite similarly to Rap1, Abf1-binding elements are essential for the full induction of RP genes but Abf1 binding to promoters does not appear to play a role in regulation of RP gene transcription (Rohde and Cardenas, 2003). Both Abf1- and Rap1-bound elements were shown to act synergistically with the downstream A/T-rich element to fully activate transcription of RP genes (Goncalves et al., 1995).

The last element, IFHL, was shown to be bound by Fhl1 (Schawalder et al., 2004; Wade et al., 2004). Like Rap1 and Abf1, Fhl1 binding is constitutive but Fhl1 then serves to recruit the Ifh1 activator via its forkhead-associated domain in a nutrient/TORC1-dependent manner (Schawalder et al., 2004; Wade et al., 2004). The slow growth phenotype of $fhl1$ mutants can be partially suppressed by Ifh1 overexpression suggesting that Ifh1 may also activate RP genes via an Fhl1-independent pathway (Cherel and Thuriaux, 1995). $IFH1$ is an essential gene, but its phenotype can be suppressed by $FHL1$ deletion, suggesting a negative role for $FHL1$ in RP genes transcription in absence of $IFH1$ (Cherel and Thuriaux, 1995). Consistent with this prediction, TORC1 and PKA were shown to impinge on Ifh1/Fhl1 activity by regulating the repressor Crf1, which competes with Ifh1 for binding of Fhl1 and represses transcription (Martin et al., 2004). Mechanistically, TORC1 inhibition results in the activation of the Yak1 kinase, which phosphorylates Crf1, thereby triggering its nuclear accumulation and its repressive activity on RP genes.

Ribosome biogenesis genes

The $ribi$ regulon is not as well defined as the $RP$ regulon. Although it could be strictly functionally defined as genes whose products participate in the biosynthesis of ribosomes, the definition used in this work is the one proposed by Wade and colleagues (Wade et al., 2006). They defined the $ribi$ regulon as a set of genes regulated with similar kinetics and/or intensities across various conditions which primarily contains genes functioning in ribosome biogenesis and translation. The distinction
mainly lies in the inclusion of genes that do not function in the biosynthesis of ribosomes. The caveat of this definition is that some RP genes could fall in the ribi regulon and that some genes regulated by TORC1 and functioning in ribosome biogenesis will not be included because of their specific kinetics. However, by definition, only genes that are regulated in a similar manner and that should therefore share common promoter structures and/or common regulators will be included.

Promoter structure – PAC and RRPE elements

Two conserved elements were found to be highly enriched at distinct positions in ribi gene promoters. A proximal element was named after the type of genes in whose promoters it was first found: the PAC element (Polymerase A (I) and C (III); Figure 5A). The second more distal element was named after the whole regulon: Ribosomal RNA Processing Element (RRPE; Figure 5B). Both elements are often found in the promoters of the same genes (Wade et al., 2006). Trans-acting factors binding to those elements were only recently discovered and are described in the following sections.

Figure 5. Consensus sequences of PAC (A) and RRPE (B) elements (Wade et al., 2006).

Sth3 binds to RRPE elements

Sth3 was first identified as an in vitro RRPE-binding protein in a large scale screen based on electrophoretic mobility shift assays (EMSA) and was shown to bind to the promoters of RRPE-containing ribi genes in vivo (Liko et al., 2007). Interestingly, Sth3 does not have any known DNA-binding domain and could account for a new class of DNA-interacting factor. Sth3 was shown to associate to ribi gene promoters and to be necessary for the full induction of ribi and RP genes upon glucose readdition in quiescent cells (Liko et al., 2007). These data suggest that Sth3 is a transcriptional activator. However, overexpression studies by the same group recently suggested that Sth3 acts as a ribi/RP genes repressor in exponentially growing cells (Liko et al., 2010).
Interestingly, Stb3 (Sin Three Binding protein 3) was first identified as a protein interacting with Sin3, which is part of the Rpd3L complex, a known repressor complex of ribi/RP genes (Humphrey et al., 2004; Kasten and Stillman, 1997; Rohde and Cardenas, 2003) (see Rpd3, a class I histone deacetylase). However, disruption of neither Sin3 nor Rpd3 attenuates the growth phenotype caused by Stb3 overexpression, which is only suppressed by the deletion of yet another histone deacetylase, HOS2, the catalytic subunit of the Set3 chromatin remodeling complex (Liko et al., 2010). Oddly, Hos2 does not seem to play a role in ribi/RP genes regulation downstream of TORC1, while Rpd3 mediates most of it (Humphrey et al., 2004). These discrepancies still need to be addressed.

**Dot6 and Tod6 bind to PAC elements to repress ribi genes**

Even more recently, Dot6 and Tod6 were identified as transcription factors binding to PAC elements in three independent large scale screens. They were first identified according to their consensus binding sequence determined with short DNA duplex arrays (Badis et al., 2008; Zhu et al., 2009) and Dot6 was isolated from a phage display library which was selected for binding activity to a PAC motif (Freckleton et al., 2009).

Dot6 and Tod6 are similar helix-turn-helix transcription factors. Their DNA-binding domain most resemble those of the SANT family (Aasland et al., 1996). Both proteins are necessary for the downregulation of ribi genes and are recruited to their promoters upon stresses, such as heat shock and glucose starvation, or upon TORC1, Sch9 or PKA inhibition (Freckleton et al., 2009; Lippman and Broach, 2009; Zhu et al., 2009). The signals from TORC1, Sch9 and PKA to promote ribi gene expression were shown to depend to varying degrees on Dot6 and Tod6: TORC1 interfering more with Tod6 repressive activity, Sch9 downregulating both factors nearly equally and PKA interfering more with Dot6 (Lippman and Broach, 2009). Interestingly, Tod6 is itself a ribi gene and has a PAC motif in its promoter (Wade et al., 2006), which is suggestive of a negative feedback mechanism. Of note, Dot6 and Tod6 also repress RP genes transcription, however it is not clear whether they act directly at RP promoters or whether the RP gene response is an indirect effect of ribi gene regulation (Lippman and Broach, 2009).
Interestingly, Dot6 and Tod6 were found to interact with the RPD3L complex (Shevchenko et al., 2008) (see Rpd3, a class I histone deacetylase). The fact that Dot6 and Tod6 function as repressors of transcription is in good agreement with this observation and would suggest that both proteins serve to recruit the RPD3L complex to *ribi* gene promoters.

Dot6 was previously identified as a high-copy disruptor of transcriptional silencing at the telomeres, the mating type locus, and the rDNA (Singer et al., 1998). Although the mechanism was never studied in detail, this effect could be linked to its interaction with the RPD3L complex which is known to play a negative role in these processes (Sun and Hampsey, 1999; Vannier et al., 1996). However RPD3L has a negative function in transcriptional silencing; therefore Dot6 overexpression probably does not lead to the impairment of RPD3L functions by a simple titration mechanism. Rather Dot6 overexpression would be predicted to enhance RPD3L function in silencing inhibition.

Regulators of both *ribi* and *RP* regulons

*Sfp1, a transcription factor and direct substrate of TORC1*

*Sfp1* was first identified in 1991 as a Zinc-finger transcription factor whose deletion induced a very strong growth phenotype (Blumberg and Silver, 1991). It was later found in that, in addition to a slow growth phenotype, *sfp1* cells have a very small size, about one third of the size of *wt* cells which makes it the smallest viable deletion mutant in budding yeast (Jorgensen et al., 2002; Xu and Norris, 1998). Ribosome biogenesis has been identified as a central mechanism in cell size determination (see *G1 progression, mass accumulation and cell size*), and *Sfp1* was therefore tested for its possible implication in that process. Both its deletion and its exclusion from the nucleus were indeed shown to induce the transcriptional repression of *ribi* and *RP* genes (Jorgensen et al., 2002; Jorgensen et al., 2004). Conversely, overexpression of *Sfp1* leads to the subsequent hyperactivation of the *ribi* regulon quickly followed by the *RP* regulon (Jorgensen et al., 2002; Jorgensen et al., 2004). This observation would suggest that *Sfp1* primarily regulates the *ribi* regulon and that *RP* genes are upregulated as an indirect result of elevated ribosome biosynthesis levels. However, parallel large-scale studies of yeast transcription factors binding to the chromatin showed that *Sfp1* binds to *RP* genes promoters in
rapidly growing cells while it could not be detected at \textit{ribi} genes (Lee et al., 2002). The exact mechanism and hierarchy of transcriptional regulation by Sfp1 is still not well understood, but this apparent discrepancy in \textit{ribi} and \textit{RP} gene regulation may be reconciled in a model involving parallel regulation mechanisms.

Sfp1 was shown to regulate the expression of \textit{ribi} gene reporter constructs in an RRPE-dependent manner, although direct binding to that element could never be observed in ChIP experiments (Fingerman et al., 2003). It is also unclear where it exactly binds in \textit{RP} gene promoters. Interestingly, the Sfp1 protein was recently shown to have intrinsic DNA binding specificity for an RRPE-like A/T-rich motif \textit{in vitro} (AAAAWTTTT) (Zhu et al., 2009), which suggests that Sfp1 indeed targets this element directly, but, for reasons not understood, cannot be detected \textit{in vivo}.

Shifting yeast cells to a non-fermentable medium or treating them with rapamycin induces Sfp1 relocation from the nucleus to the cytoplasm (Lempiainen et al., 2009; Singh and Tyers, 2009). In addition, Sfp1 is directly phosphorylated by TORC1 \textit{in vitro} and its phosphorylation is downregulated by rapamycin treatment \textit{in vivo} (Lempiainen et al., 2009). The substitution of the serines phosphorylated by TORC1 with non-phosphorylatable alanines yields a hypomorphic \textit{SFP1} variant which mainly resides in the cytoplasm (Lempiainen et al., 2009). These observations suggest that TORC1 promotes Sfp1 activity by phosphorylating it and thereby inducing its nuclear accumulation.

Mechanistically, the Shore and Tyers groups have shown that Sfp1 nuclear vs. cytoplasmic localization depends on an essential Rab escort protein, Mrs6, which usually functions in vesicular trafficking (Lempiainen et al., 2009; Singh and Tyers, 2009). However, these groups have published conflicting results regarding a positive or negative role of Mrs6 in Sfp1 accumulation in the nucleus.

Importantly, disruption of \textit{SFP1} causes a growth phenotype that is additive to the one of Sch9, which strongly argues that they act in parallel pathways downstream of TORC1 to promote \textit{ribi/RP} genes expression (Jorgensen et al., 2004; Lempiainen et al., 2009).
Rpd3, a class I histone deacetylase

Rpd3 is a class I histone deacetylase which functions in various cell processes. The Rpd3 enzyme acts in two distinct complexes, RPD3S and RPD3L, which share Sin3 and Ume1 as common subunits but can be resolved by gel filtration thanks to their specific subunits (Carrozza et al., 2005; Yang and Seto, 2008). RPD3S contains Rco1 and Eaf3, the latter being shared with the NuA4 acetyltransferase complex. RPD3S functions as a repressor of spurious transcription initiation that occurs inside active ORFs (Carrozza et al., 2005).

RPD3L is larger and has been reported to contain many different subunits including Sap30, Sds3 and Pho23 (Lechner et al., 2000; Loewith et al., 2001; Shevchenko et al., 2008; Zhang et al., 1998). Sap30 and Sds3 are thought to be essential core components of RPD3L (Lechner et al., 2000; Zhang et al., 1998), while other interacting partners of RPD3L might only function for some specific activities of the complex and/or interact in a more labile manner.

In addition to its role in the regulation of the rDNA chromatin, Rpd3 is required for proper repression of ribi/RP genes upon rapamycin treatment (Humphrey et al., 2004; Rohde and Cardenas, 2003). However, the two studies showed conflicting results about the mechanism of signal transduction from TORC1 to the acetylation of histones at these promoters. The first study argued for a more pronounced regulation of the binding of Esa1, the catalytic subunit of the counteracting NuA4 acetyltransferase complex, to the RP genes promoters rather than a regulation of Rpd3 which showed no significant change in occupancy at the same loci (Rohde and Cardenas, 2003). Contrastingly, the second study could detect clear changes in Rpd3 binding in ChIP on ChIP and could confirm these observations in ChIP-qPCR experiments, but a possible role of Esa1 or other acetyltransferases was not investigated (Humphrey et al., 2004).

RNA Polymerase III synthesizes the 5S rRNA and tRNAs

RNA Pol III is in charge of synthesizing many different types of non-coding transcripts from genes with different promoter structures. In yeast, RNA Pol III transcribes the 5S rRNA (encoded in rDNA
repeats; Figure 4A), the tRNAs, the 7SL RNA (a component of the signal recognition particle), the RNase P RNA, the U6 snRNA and the snR52 snoRNA (Dieci et al., 2007).

Two types of promoters and their initiation factors

RNA Pol III-transcribed genes have special types of promoters whose elements are positioned downstream of the transcriptional start sites in the transcribed sequence (Willis, 1993). Two types of RNA Pol III promoters can be distinguished in budding yeast. A third type of promoter which resembles RNA Pol II upstream promoters exists in metazoans but not S. cerevisiae. The first type of promoter (type I) is unique to the 5S rRNA gene and comprises three elements: an A box, an intermediate element and a C box separated by sequences of specific lengths. Other RNA Pol III-transcribed genes are controlled by type II promoters composed of two elements, an A box (similar to the A box in type I promoters) close to the promoter and a B box separated by a spacer whose length is variable (Geiduschek and Tocchini-Valentini, 1988).

The two promoter types have distinct mechanisms of transcription initiation (Geiduschek and Tocchini-Valentini, 1988). Initiation from type I promoters requires all three RNA Pol III transcription initiation factors (TFIILIA-C). TFIILIA binds to the intermediate element and to the C box and helps to recruit TFIIC which interacts also with the A box. Type 2 promoters do not require TFIILIA. The presence of both A and B boxes is sufficient to recruit TFIIC. Central to the initiation process, TFIIB is then recruited by TFIIC upstream of the promoter and interacts with RNA Pol III to form the preinitiation complex.

Maf1, a conserved RNA Pol III repressor

Regulation of RNA Pol III by TORC1 depends on the conserved protein Maf1 which was first identified for its phenotype in SUP11-mediated non-sense codon suppression (Boguta et al., 1997; Murawski et al., 1994). Maf1 was later shown to inhibit RNA Pol III and to downregulate tRNA production according to growth conditions (Pluta et al., 2001; Upadhya et al., 2002). Maf1 is a phosphoprotein in rapidly growing cells and becomes dephosphorylated upon TORC1 inhibition by rapamycin or application of various stresses such as nitrogen/carbon starvation, heat shock, osmotic
stress and DNA damage (Moir et al., 2006; Oficjalska-Pham et al., 2006; Roberts et al., 2006). Once dephosphorylated, Maf1 interacts with RNA Pol III and downregulates its activity (Oficjalska-Pham et al., 2006; Roberts et al., 2006). Maf1 was also shown to interact with TFIIIB in biochemical studies and was proposed to inhibit both the recruitment of TFIIIB to the chromatin and its activity when already bound to RNA Pol III genes promoters (Desai et al., 2005). These observations fit with the observation that Maf1 is recruited to RNA Pol III genes in vivo under repressing conditions (Oficjalska-Pham et al., 2006; Roberts et al., 2006). The exact contribution of both interactions to RNA Pol III repression has not been fully evaluated yet.

Other levels of Maf1 regulation might contribute to the fine-tuning of its activity. Maf1 localization was shown to be regulated by TORC1, as rapamycin treatment induces its accumulation in the nucleus, in part via the regulation of its phosphorylation (Moir et al., 2006). This mechanism is however not critical to Maf1 regulation as mutants deleted for MSN5 (which encodes a Maf1-interacting nuclear exportin) show constitutive Maf1 nuclear localization but no evident phenotype in RNA Pol III regulation (Towpik et al., 2008). Very recently, Maf1 subnuclear localization was proposed as a regulatory step as Maf1 does not penetrate the nucleolus until cells are treated with rapamycin (Wei et al., 2009).

TORC1 was proposed to regulate Maf1 phosphorylation via the PP2A phosphatases, as disruption of their activity by mutating their catalytic subunits prevents Maf1 dephosphorylation upon rapamycin treatment (Oficjalska-Pham et al., 2006; Roberts et al., 2006). It is not clear though whether TORC1 actually modulates the activity of PP2A phosphatases towards Maf1 or if another mechanism is at play and is just masked by their disruption. TORC1 was also proposed to directly phosphorylate Maf1 (Wei et al., 2009). However the phosphorylation sites were not mapped and TORC1 activity toward Maf1 in in vitro assays was very weak (Wei et al., 2009; Wei and Zheng, 2009).

Maf1 was independently shown to be phosphorylated by PKA on R[R/K]xS* motifs which fit the preferred consensus sequence of the kinase (Moir et al., 2006). Notably, hyperactivation of PKA by deletion of its regulatory subunit Bcy1 or expression of the activated RAS GTPase leads to Maf1
hyperphosphorylation in conditions where it is normally dephosphorylated (Moir et al., 2006). However, TORC1 inhibition still leads to the downregulation of RNA Pol III in those mutants suggesting that TORC1 acts in parallel of PKA (Moir et al., 2006).

**Crosstalks between RNA Polymerases**

Ribosomal components must be produced in equimolar levels for efficient synthesis of ribosomes. How this is achieved is still far from being understood. Some mechanisms are obvious, such as the production of the 25S, 18S and 5.8S rRNAs as a single precursor transcript, but how the different RNA polymerases are coordinated to produce equimolar levels of ribosomal proteins and 5S rRNA is still largely misunderstood. A significant increase in our understanding of this problem was realized with the functional uncoupling of TORC1 inhibition and RNA Pol I downregulation. Overexpression of Rrn3 fused to a subunit of RNA Pol I mimicks a constitutively active polymerase that is resistant to rapamycin (Laferte et al., 2006). Interestingly, in addition to 35S rRNA transcription, 5s rRNA and *RP* gene transcription also remains derepressed upon rapamycin treatment in these cells and consequently, ribosome biosynthesis is partially resistant to TORC1 inhibition. Regulation of RNA Polymerases is therefore not achieved only by parallel regulatory mechanisms and it is likely that signals in series are also at play.

**Ribosome biogenesis homeostasis and negative feedback on TORC1**

Interfering with the function of specific *ribi* genes has been shown to lead to a hyperactivation of the whole regulon suggesting a negative transcriptional feedback loop (Mnaimneh et al., 2004). Interestingly, deletion of the *ribi* activator *SFP1* leads to TORC1 hyperactivation suggesting that the negative feedback loop might involve TORC1 itself (Lempainen et al., 2009). TORC1 has also been shown to be hyperactivated upon inhibition of translation elongation by cycloheximide (See Intracellular cues also modulate TORC1 activity) (Urban et al., 2007). This regulatory mechanism was proposed to involve amino acid accumulation (Binda et al., 2009), but has not been fully elucidated yet. It could therefore also involve ribosome biogenesis which requires translation of ribosomal proteins.
G1 progression, mass accumulation and cell size

Cell size, either defined as the volume of the cell or their biomass content, is determined by two antagonistic processes: growth and cell division. In budding yeast, the cell size is mainly determined in early G1 as cells need to achieve a critical size determined by nutrient availability in order to commit to division and enter S phase (Jorgensen and Tyers, 2004; Rupes, 2002). This mechanism is robust as evidenced by its resistance to profound perturbations such as partial translation inhibition by cycloheximide (Jorgensen et al., 2004). In contrast, cell size determination was shown to be very sensitive to the rate of ribosome biogenesis as mutants impaired in ribi gene regulation, such as SCH9 or SFP1 deletion strains, have a small cell size phenotype (Jorgensen et al., 2002; Jorgensen et al., 2004). To some extent, inhibition of specific ribi gene transcription recapitulates this phenotype (Bernstein et al., 2007; Mnaimneh et al., 2004). As ribosome biogenesis is tightly regulated according to environmental conditions it is thought to be the main process coupling nutrient availability to cell size determination (Jorgensen and Tyers, 2004) (Figure 6).

![Diagram of ribosome biogenesis and cell size regulation](image)

**Figure 6.** Simplified scheme of ribosome biogenesis links to cell size regulation

How ribosome biogenesis is sensed to regulate cell size is not entirely clear yet. In addition, its relationships with cell cycle progression were shown to be more complex than previously anticipated in studies involving the U3 snoRNP. Depletion of the protein subunits of the U3 snoRNP by placing them under the control of glucose-repressible promoters leads to a cell cycle arrest in G1, indicating that ribosome biogenesis may also play a negative role in progression through that phase of the cycle.
(Bernstein and Baserga, 2004). Interestingly, this effect appears before any obvious phenotype in growth or 18S rRNA levels suggesting that some steps of the ribosome biogenesis process itself rather than the levels of ribosomes are regulating progression through G1 (Bernstein et al., 2007).

**Research project**

Given the many gaps in our understanding of the TORC1 signaling network in *S. cerevisiae*, the first goal of our work was to identify new targets of TORC1 and Sch9, the latter of which had just been characterized as being directly phosphorylated and thereby activated by TORC1 (Urban et al., 2007). In a second part, we sought to characterize in more detail the regulation of these novel targets as well as their function downstream of TORC1 and/or Sch9.
II. Articles and results

Sch9 is directly phosphorylated and activated by TORC1 (1st article)

Sch9 was known to have similar functions as TORC1 in translation initiation and transcriptional regulation of ribi/RP genes and stress/starvation response genes. In mammals, the p70 S6 kinases were previously shown to be directly phosphorylated by mTORC1 on C-terminal hydrophobic motifs. Based on Sch9 functional and structural similarities with TORC1 and p70 S6 kinases respectively, Sch9 was hypothesized to be directly phosphorylated by TORC1 (Urban et al., 2007).

Purified Sch9 was shown to be indeed directly phosphorylated by TORC1 at its C-terminus in vitro. In living cells, Sch9 C-terminal phosphorylation is sensitive to rapamycin. Phosphorylation was mapped to at least five serine and threonine residues (Two SP/TP sites or turn motifs and three hydrophobic motifs). Evidence was provided that Sch9 activity was inhibited upon TORC1 inhibition by rapamycin. By replacing some or all of the TORC1 phosphorylation sites with negatively charged amino acids (Asp/Glu) to mimick constitutive phosphorylation, alleles of SCH9 (SCH93E or SCH9DE respectively) whose activity was no longer sensitive to rapamycin could be generated. Using those alleles, Sch9 was shown to partially mediate TORC1 signals to stimulate translation initiation, cell cycle progression and ribi/RP genes expression and to inhibit accumulation of glycogen.

Personal participation to the article:

- Figure 3B: in vitro kinase assays with immunoprecipitated Sch9 mutants on synthetic peptide substrates
- Figure 7C: in vitro kinase assays with purified Sch9 and purified recombinant Rps6
Sch9 Is a Major Target of TORC1 in Saccharomyces cerevisiae

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SUMMARY

The Target of Rapamycin (TOR) protein is a Ser/Thr kinase that functions in two distinct multi-protein complexes: TORC1 and TORC2. These conserved complexes regulate many different aspects of cell growth in response to intracellular and extracellular cues. Here we report that the AGC kinase Sch9 is a substrate of yeast TORC1. Six amino acids in the C terminus of Sch9 are directly phosphorylated by TORC1. Phosphorylation of these residues is lost upon rapamycin treatment as well as carbon or nitrogen starvation and transiently reduced following application of osmotic, oxidative, or thermal stress. TORC1-dependent phosphorylation is required for Sch9 activity, and replacement of residues phosphorylated by TORC1 with Asp/Glu renders Sch9 activity TORC1 independent. Sch9 is required for TORC1 to properly regulate ribosome biogenesis, translation initiation, and entry into G0 phase, but not expression of Gln3-dependent genes. Our results suggest that Sch9 functions analogously to the mammalian TORC1 substrate S6K1 rather than the mTORC2 substrate PKB/Akt.

INTRODUCTION

In eukaryotes two distinct, conserved, multiprotein complexes known as TOR complex 1 (TORC1) and TORC2 function as major regulators of cell growth (Wullschleger et al., 2006). In both complexes, Target of Rapamycin (TOR), a large Ser/Thr protein kinase belonging to the family of phosphatidylinositol kinase-related kinases (Keith and Schreiber, 1995), functions as the catalytic subunit. TORC1, but not TORC2, is directly inhibited by the macrocyclic lactone rapamycin (Jacinto et al., 2004; Loeewith et al., 2002; Sarbassov et al., 2004).

S. cerevisiae TORC1 contains Lst8, Kog1, Tco89, and either Tor1 or Tor2 (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003). Based primarily on the observed phenotypic similarities between rapamycin-treated and nutrient-starved cells it is generally believed that TORC1 couples nutrient cues to the cell growth machinery (Rothe et al., 2001). TORC1 activity also appears to be influenced by a number of other noxious stresses and, in mezoans, by extracellular mitogens (Crespo et al., 2001; Kim et al., 2002; Sarbassov and Sabatini, 2005). Under favorable conditions, yeast TORC1 promotes growth by stimulating translation initiation and, via transcription factors such as Lfhl1, Cre1, and Sfp1, expression of genes required for synthesis and assembly of the translation machinery. In addition to promoting anabolic processes, TORC1 also antagonizes entry into G0 phase, the induction of stress response programs, and catabolic processes including autophagy and expression of gene products required for the metabolism of nonpreferred nutrients. Again, TORC1 regulates many of these processes by influencing the localization/activity of transcription factors including Gln3 (nitrogen discrimination pathway), Rtg1/Rtg3 (retrograde signaling), and Msn2/Msn4 (stress response) (De Virgilio and Loeewith, 2006).

How TORC1 activity is linked to its diverse downstream targets is not well understood. Several processes are thought to be regulated through modulation of protein phosphatase 2A activity; however, the direct link between TORC1 and the phosphatases has not been clearly defined (Duvel et al., 2003). The lack of well-characterized TORC1 substrates with defined phosphorylation sites has also hampered the identification of physiological stimuli and upstream regulatory components that control TORC1 activity.

Activity of many members of the AGC protein kinase family (homologous to protein kinases A, G, and C)
requires both phosphorylation of the kinase domain activation
loop by PDK1 and binding of the conserved “hydrophobic motif” (HM; F-X-X-F/Y/S/T-F/Y) found C terminal of the catalytic domain to a pocket in the kinase domain. The latter often depends on HM phosphorylation (Gold et al., 2006).

Mounting evidence suggests that TOR complexes phosphorylate the HM of several AGC kinases. In vivo and in vitro data show that mammalian TORC1 (mTORC1) and mTORC2 phosphorylate the HM in S6K1 and PKB/Akt, respectively (Burnett et al., 1998; Itoh et al., 1999; Sarbassov et al., 2005). S. cerevisiae TORC2 and a Schizosaccharomyces pombe TOR complex have also been reported to phosphorylate the HM in the AGC kinases Ypk1/2 and Gad8, respectively (Kamada et al., 2005; Matsuo et al., 2003). Both mammalian and yeast TOR complexes phosphorylate additional residues adjacent to the HM, including a sequence termed the “turn motif,” which are often followed by a Pro (Kamada et al., 2005; Matsuo et al., 2003; Montagne and Thomas, 2004). Phosphorylation of the turn motif is thought to further stabilize the interaction between the HM and its binding pocket (Gold et al., 2006).

In this study we queried whether the yeast AGC kinase Sch9 could be a direct substrate for TORC1. Previous studies have revealed phenotypic similarities between sch9 cells and rapamycin-treated cells including nuclear localization and activation of the Rim15 kinase and decreased expression of genes encoding proteins required for ribosome biogenesis (Jorgensen et al., 2004; Pedruzzi et al., 2003). Furthermore, tor1 and sch9 cells also share increased lifespan (Kaeberlein et al., 2005). Last, a recent report suggested that Sch9 becomes partially dephosphorylated upon rapamycin treatment (Jorgensen et al., 2004).

Here we show that Sch9 is directly phosphorylated by TORC1 at multiple C-terminal sites and by the yeast PDK1 ortholog in the activation loop. Both phosphorylation events are independently required for Sch9 activity. Phosphorylation of TORC1 sites is abolished under either nitrogen or carbon starvation and transiently reduced when cells are subjected to various stress conditions. These observations support the notion that TORC1 activity is regulated by nutrient abundance and inhibited by noxious stress. Using TORC1-independent versions of Sch9, we found that Sch9 is a major effector of TORC1 that appears to function similarly to the mTORC1 substate S6K1.

RESULTS

Chemical Fragmentation Reveals Multiple Rapamycin-Sensitive Phosphorylation Sites in the Sch9 C Terminus

Analysis of Sch9 phosphorylation using SDS-PAGE migration shifts has been complicated by both the large size of Sch9 (~100 kDa) and by the presence of multiple phosphorylation sites. To circumvent these challenges we tested various chemical reagents used for fragmentation of proteins (Burgess et al., 2000), of which NTCB (2-nitro-5-thiocyanatobenzoic acid) proved to be particularly useful. NTCB selectively cyanoates Cys residues, and under alkaline conditions this is followed by chain cleavage at the modified residues. This allowed us to analyze smaller fragments that contained fewer phosphorylated residues and were better resolved by immunoblotting. Treatment of C-terminally HA-tagged Sch9 in a crude yeast extract yielded a highly reproducible cleavage at some of the nine cysteines found in Sch9 (Figure 1A).

Phosphorylation of Sch9 was decreased in cells treated with rapamycin (Rap) or wortmannin (WM) but was increased in the presence of a sublethal dose of cycloheximide (CHX). Cleavage with NTCB revealed that this included a dephosphorylation or hyperphosphorylation of the Sch9 C terminus, respectively (Figure 1A). Treatment with λ phosphatase showed that the ladder of bands migrating around 50 kDa in SDS-PAGE represents multiple phosphoisoforms of the same fragmentation product (Figure 1B). This was subsequently found to include the activation loop phosphorylation site T570 (Liu et al., 2005) and thus probably encompasses amino acids 554–824 of Sch9 and the SHA tag (see Figures 2A and 2D).

Next we tested whether physiological conditions predicted to regulate TORC1 activity also affect the phosphorylation of the Sch9 C terminus. Shifting cells from a medium containing glucose and Gln to media lacking either a carbon or nitrogen source caused a rapid dephosphorylation that was quickly reversed upon readdition of the missing nutrient. The centrifugation step required for the medium change also led to a transient partial dephosphorylation of the Sch9 C terminus (Figure 1C). Changing the nitrogen source from NH4+ to urea resulted in a rapid dephosphorylation followed by a complete repophosphorylation in less than 2 hr (data not shown). Transferring cells grown in a low-phosphate medium into a phosphate-free medium also caused a dephosphorylation, although with much slower kinetics (Figure 1D). Again, this was quickly reversed upon phosphate readdition. Further analysis showed that the C terminus of Sch9 was transiently dephosphorylated when cells were subjected to various stress conditions, including high salt, redox stress, or a shift to a higher temperature (Figure 1E).

Next wished to determine whether the extent of Sch9 C-terminal phosphorylation correlated with nutrient quality (Figure 1F). Sch9 was found to be slightly less phosphorylated when cells were supplied with raffinose or ethanol plus glycerol compared to glucose or galactose as carbon sources. NH4+- and Pro-based media also supported slightly less phosphorylation compared to Gln- or urea-based media. However, the extent of Sch9 phosphorylation did not always correlate with growth rate; cells grew faster in NH4+ versus Pro-based medium, but Sch9 C-terminal phosphorylation was similar under both conditions.
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TORC1 Phosphorylates Six Residues in the C Terminus of Sch9

To identify the sites phosphorylated in Sch9, we purified Sch9 from actively growing yeast and mapped potential phosphorylation sites by mass spectrometry (see Table S3 in the Supplemental Data available with this article online). Building on these results, we started an extensive mutational analysis that identified seven Ser/Thr residues in the C terminus that when changed to Ala caused obvious alterations in SDS-PAGE migration of fragmented Sch9 (Figure 2A). Cumulative substitution of these Ser/Thr residues to Ala led to a progressive loss of phosphorylated species, and a version of Sch9 lacking all seven sites yielded a C-terminal fragment upon NTCB cleavage that migrated as a single band (Figure 2B; uncropped image, Figure S1).

Experiments with a variety of constructs containing multiple Ser/Thr to Ala substitutions generally suggested that the various sites can be phosphorylated independently; only the phosphorylation of T723 seemed to depend to some extent on prior phosphorylation of S726 (data not shown). With the exception of T570 in the activation loop, phosphorylation of the remaining six C-terminal sites was sensitive to rapamycin treatment (Figure 2C, see also Figure 4A) demonstrating that it occurred in a TORC1-dependent manner.

Figure 2D shows the domain structure of Sch9 and the position of the phosphorylated residues in the C-terminal fragment. In addition to T570, the sites that were identified included T737 in the classical HM of AGC kinases and two Ser/Thr-Pro sites, T723 and S726. Two more sites were found in the C-terminal extension of the C terminus beyond the HM of Sch9 (S758 and S765). These show similarity to the HM, especially the presence of bulky hydrophobic residues at positions +4, +1, and +2. Finally, S711 was found to be phosphorylated as well. This residue is also followed by two hydrophobic amino acids but is not preceded by a hydrophobic residue at position +4.

To test whether Sch9 is a direct substrate for TORC1, we first queried whether TORC1 components can physically interact with Sch9. Although we were unable to coimmunoprecipitate TORC1 with Sch9, we detected a weak interaction between Tor1 and Sch9 using a two-hybrid approach (data not shown). Next we asked whether Sch9 is a substrate for TORC1 in vitro. Indeed, TORC1 purified from yeast phosphorylated recombinant Sch9 (Figure 2E). This phosphorylation was strongly diminished if TORC1 was obtained from cells treated with rapamycin or from cells expressing only a catalytically inactive version of Tor1 (Tor110275A). Recombinant Sch9 lacking all six C-terminal sites (Sch9Δ6) was much less phosphorylated compared to Tor1-3A-723A.

The Sch9 C terminus becomes temporarily dephosphorylated in response to high salt, redox, and temperature stress.

(F) Phosphorylation of the Sch9 C terminus is reduced in cells growing on less preferred nitrogen or carbon sources. (A–F) show anti-HA immunoblots of untreated (A and B) and NTCB-treated (A–F) protein extracts obtained from WT cells containing a plasmid-based copy of SCH9:HA. Only the C-terminal fragment is shown in (G–F).
to Sch9WT, suggesting that we have identified the majority of the residues in Sch9 that are modified by TORC1.

Phosphorylation of Sch9 was specific to TORC1: purified TORC2, although able to phosphorylate a physiological substrate, Ypk2 (Kamada et al., 2005), was unable to phosphorylate Sch9 in vitro (Figure S2).

To determine whether each of the six sites in the Sch9 C terminus could be phosphorylated by TORC1, we “added back” Ser/Thr residues to the Sch9WT mutant and asked whether this improved their phosphorylation. A comparison between Sch9WT and a version containing S711 (Sch9SA) showed that S711 was poorly phosphorylated in the in vitro assay (Figure 2E). Sch9 versions containing any of the other sites in addition to S711 were more strongly phosphorylated than Sch9SA, indicating that at least five sites in the Sch9 C terminus can be directly phosphorylated by TORC1 (Figure 2F). Among these, the HM-like sites S758 and S765 in the CE appeared to be particularly good substrates in vitro while the Ser/Thr-Pro sites T723 and S726 were less used.

**TORC1 Phosphorylation Sites Are Critical to Sch9 Function**

To analyze the importance of TORC1-dependent phosphorylation of Sch9 in vivo, we took advantage of the observation that sch9 cells grew slowly on YPD and not at all.
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on YPGal. Introduction of single-copy plasmids containing SCH9 or the acidic residue-substituted alleles SCH9<sup>RS</sup> (T737E, S758E, S765E) and SCH9<sup>D203E</sup> (T723D, S726D, T737E, S758E, S765E) into sch9 cells restored normal growth on both YPD and YPGal, while the Ala-substituted allele SCH9<sup>AA</sup> (T723A, S726A, T737A, S758A, S765A) failed to complement the growth defect. The SCH9<sup>RS</sup> and SCH9<sup>D203E</sup> alleles also conferred a slight resistance to rapamycin (Figure 3A).

Because deletion of GLN3 and GAT1 renders cells resistant to low doses of rapamycin (Beck and Hall, 1999), we also performed our complementation studies in a (protophor) sch9<sup>get1</sup> gln3 background. An added advantage of using this strain was that it appeared to be phenotypically more stable than sch9 cells (data not shown). Compared to sch9 cells, sch9<sup>get1</sup> gln3 cells grew markedly better on YPD and slowly on YPD + rapamycin but they still failed to grow on YPGal. Importantly, introduction of SCH9<sup>RS</sup> and SCH9<sup>D203E</sup> alleles but not of SCH9<sup>WT</sup> in this background allowed cells to grow on YPGal + rapamycin (Figure 3A). This shows that Sch9 function depends on TORC1-mediated phosphorylation of its C terminus and that substitution of the C-terminal TORC1 phosphorylation sites of Sch9 with acidic amino acids yields Sch9 proteins that appear to function independently of TORC1.

Further detailed analysis indicated that, with the possible exception of S711, all of the TORC1 phosphorylation sites in Sch9 play a positive role in Sch9 function with T737 in the HM being the most important site (Figure S3A). A version containing only a substitution of the HM site with glutamate (T737E) conferred rapamycin-resistant growth, but simultaneous replacement of several TORC1 sites with acidic residues (3E and 2D3E) resulted in a higher level of rapamycin resistance (Figure S3B).

In order to analyze how these mutations affect the Sch9 kinase activity, WT and mutated versions of Sch9-3HA were isolated from yeast cells treated with drug vehicle or rapamycin and tested for their ability to phosphorylate a peptide (GRPRTTSSFAEG; Cross et al., 1995), which is known to be phosphorylated by various AGC kinases. Sch9<sup>WT</sup> obtained from mock-treated cells was able to phosphorylate the peptide while no activity was measured when Sch9<sup>WT</sup> was isolated from rapamycin-treated cells (Figure 3D). Sch9<sup>K41A</sup> (K41A; Morano and Thiele, 1999) and Sch9<sup>D203E</sup> showed no activity toward the substrate while Sch9<sup>D203C</sup> activity was, for unknown reasons, increased by prior rapamycin treatment. Together, these results demonstrate that phosphorylation by TORC1 is necessary for both Sch9 function in vivo and catalytic activity in vitro.

Pkh Kinases Phosphorylate T570 in the Activation Loop of Sch9

Activity of many AGC kinases requires phosphorylation of a Ser/Thr residue in the activation loop by PDK kinases (Mora et al., 2004). To analyze the phosphorylation in the Sch9 activation loop, phosphospecific antibodies against phospho-T570 were generated. Immunoblotting showed that these antibodies detected Sch9<sup>WT</sup> expressed in}

sch9<sup>get1</sup> gln3 cells, but not the Ala-substituted Sch9<sup>T570A</sup> (Figure 4A). In vivo, T570 was phosphorylated similarly in Sch9<sup>WT</sup>, the inactive Sch9<sup>K41A</sup>, or the TORC1-independent versions Sch9<sup>RS</sup> and Sch9<sup>D203E</sup> as well as in Sch9<sup>Wt</sup> after rapamycin treatment (Figure 4A). This suggests that phosphorylation of the HM is not required to facilitate subsequent phosphorylation of the activation loop. The finding that both Sch9<sup>T570A</sup> and Sch9<sup>AA</sup> are inactive (see Figure 3B), although they still are phosphorylated by TORC1 and Pkh kinases, respectively (see Figures 2A and 4A), demonstrates that both activation loop phosphorylation and phosphorylation of the C terminus by TORC1 are independently required for Sch9 activity.

In yeast, PDKs are encoded by the PKH1 and PKH2 genes (Casamayor et al., 1999). To determine whether the activation loop in Sch9 is phosphorylated by Pkh kinases, we performed an in vitro kinase assay and found that recombinant GST-Sch9 was efficiently phosphorylated by GST-Pkh2 purified from yeast cytosol. A preparation
Figure 4. Pkh Kinases Phosphorylate the Activation Loop of Sch9
(A) Phosphospecific antibodies directed toward the activation loop specifically recognize Sch9 phosphorylated at T570. Mutating the C-terminal TORC1 sites to Ala or Asp/Glu or treating cells with rapamycin (30 min, 200 ng/ml) does not affect the phosphorylation of T570 in vivo. The indicated versions of plasmid-encoded 6HA-Sch9 were expressed in sch9Δ pkh1Δ pkh2Δ cells, and protein extracts were analyzed by immunoblotting. (B) Pkh2, but not inactive Pkh2<sup>C208R</sup>, phosphorylates recombinant GST-Sch9 at T570 in vitro. 
(C) Phosphorylation of T570 in 6HA-Sch9, expressed from the endogenous locus, is strongly reduced in pkh1<sup>Δ</sup> pkh2<sup>Δ</sup> mutants at permissive temperature (36°C) and undetectable after a shift to nonpermissive temperature (20 min, 37°C).

of the catalytically inactive protein, Pkh2<sup>C208R</sup> (Inagaki et al., 1999), did not show any activity toward Sch9, and the activation loop mutant Sch9<sup>T570A</sup> was not a substrate for Pkh2, indicating that Pkh2 directly phosphorylates Sch9 at T570 (Figure 4B).

Immunoblot analysis showed that Pkh activity is also required for T570 phosphorylation in vivo. Relative to WT cells, at permissive temperature phosphorylation of T570 was reduced in pkh1<sup>Δ</sup> cells and strongly reduced in pkh1<sup>Δ</sup> pkh2<sup>Δ</sup> cells carrying the temperature-sensitive allele Pkh1<sup>C208Q</sup> (Inagaki et al., 1999). After incubation at nonpermissive temperature, phosphorylation of the Sch9 activation loop was undetectable in pkh1<sup>Δ</sup> pkh2<sup>Δ</sup> cells (Figure 4C). This shows that Sch9 is phosphorylated by both Pkh1 and Pkh2 in vivo.

TORC1 Is Active at the Vacuole
Sch9 was found previously to be concentrated at the vacuolar membrane, and this localization was shown to be rapamycin insensitive (Jorgensen et al., 2004). Consistently, the localization of GFP-Sch9 was not significantly altered by the introduction of mutations at TORC1 phosphorylation sites (5A, 3E, and 2D3E) (Figure 5A). We found that functional, GFP-tagged TORC1 components Tco89 and Kog1 (Figure 5A and data not shown) also localized to the vacuolar membrane. Although the localization of TORC1 is currently debatable, the existence of a pool of TORC1 at the vacuolar membrane is consistent with several reports (discussed in De Virgilio and Loewith, 2009).

Thus, we wished to corroborate our localization data and determine whether TORC1 is active at the vacuole.
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To test directly whether TORC1 can phosphorylate Sch9 at the vacuole, we fused the C-terminal portion of Sch9 (cSch9 = aa 709–824) and a tag onto the C terminus of Vac8, a palmitoylated protein that resides on the vacuolar membrane (Wang et al., 1998). Vac8-cSch9-GFP expressed in WT cells localized to the surface of the vacuole as expected (Figure 5A). Immunoblotting of untreated and NTCB-treated protein extracts showed that the C terminus of Vac8-cSch9-3HA became highly phosphorylated in a rapamycin-sensitive manner. No rapamycin-sensitive modification occurred in a construct containing alanines at all six TORC1 phosphorylation sites (Vac8-cSch9AA-3HA; Figure 5B). Further analyses showed that the TORC1 sites in this construct were hyperphosphorylated upon treatment with CHX and dephosphorylated following high salt treatment as well as carbon or nitrogen starvation (Figure 5C). Following readdition of Gin to nitrogen-starved cells, Vac8-cSch9-3HA and Sch9-5HA were rephosphorylated with similar kinetics (data not shown). Similar results were obtained when the cSch9-GFP or cSch9-3HA sequences were fused to the first 134 amino acids of Sna4, a small proteolipid of the vacuolar membrane (data not shown), confirming that our findings are not specific for Vac8. These experiments indicate that the pool of TORC1 at the vacuolar surface is active. In the future, variants of these reporter constructs may be useful to probe for TORC1 activity at other loci.

TORC1 Regulates the Ribi and RP Regulons in Part via Sch9
To begin to investigate which of the many different TORC1 readouts are mediated by Sch9, we used global transcriptional analysis to compare the rapamycin response of WT cells (W303) expressing plasmid-encoded Sch9WT(WT/WT) with those expressing plasmid-encoded Sch9D3E(WT/2D3E). We hypothesized that TORC1-independent Sch9D3E should act in a dominant manner to attenuate the response of genes that are regulated by TORC1 via Sch9. An analysis of all genes whose expression changed at least 3-fold after rapamycin addition revealed a bi-phasic response to rapamycin, with one set of genes responding maximally after 20 or 30 min and a second, essentially nonoverlapping set of genes responding maximally at later time points (Figure S4).

Thus we separately analyzed early- and late-responsive genes whose expression changed at least 3-fold in WT/WT cells (Figure 6A). At early time points following plasmid-encoded Sch9D3E, in cells expressing Sch9AA, Rim15 localizes constitutively to the nucleus.

(C) FACS analysis of sch9 cells containing the indicated plasmid-based alleles of Sch9 following treatment with rapamycin (4 hr, 200 ng/ml) or drug vehicle. Expression of Sch9D3E prevents the rapamycin-induced cell-cycle arrest, observed as accumulation of cells with a 1n DNA content. An enriched portion of cells expressing Sch9AA have a 1n DNA content even in the absence of rapamycin.

(D) sch9 gat7 gin3 cells expressing Sch9WT or Sch9D3E show a reduced glycerol accumulation upon rapamycin treatment (6 hr, 200 ng/ml) compared to cells expressing Sch9WT.

Figure 6. Sch9 Is Required for TORC1 to Properly Regulate Transcription and Entry into G2 Phase
(A) Microarray analysis comparing WT cells (W303) containing a plasmid-based copy of SCH9WT(WT/WT) or SCH9D3E(WT/2D3E) uncovers the Sch9 dependence of rapamycin-induced changes in transcription. Genes showing at least 3-fold change in expression were classified as either early- or late-responsive by comparing the rapamycin response in WT/WT cells at early (20/30 min) and late (90/120 min) time points. Genes were sorted according to the maximal Sch9 dependence of their response (df = log2(WT/2D3E) – log2(WT/WT)). For genes above the black line the rapamycin-induced change in expression is diminished ≥ 2-fold in WT/2D3E compared to WT/WT cells (i.e., df ≥ 1 or < –1). Ribosome biosynthesis genes and genes encoding RPs as well as genes, whose expression is regulated by Gin3/Gat1 or Msn2/4, are indicated. Color codes show the log2 of the expression change relative to untreated cells (green-red) and the corresponding df value (blue-gold).
(B) The rapamycin-induced (200 ng/ml, 30 min) translocation of GFP-Rim15GFP TreB into the nucleus is blocked in sch9 rim15 cells expressing...
rapamycin treatment the expression of 272 genes was reduced 3-fold. Repression of a majority (181) of these genes was attenuated at least 2-fold in WT/2D3E cells compared to WT/WT cells, suggesting that TORC1-dependent phosphorylation of Sch9 contributes to the regulation of these genes. This group included predominantly genes that encode factors involved in the synthesis of ribosomes, tRNAs, and nucleotides (SGD), most of which have previously been assigned to the ribosome biogenesis (Ribi) regulon (Jorgensen et al., 2004). Quantitative PCR analyses confirmed that the effect of rapamycin on the expression of several Ribi genes (PWP1, UTP13, DIP2, and CIC1) was reduced in WT/2D3E compared to WT/WT cells (Figure S5A). Further experiments in the TB50 genetic background showed that the effect of rapamycin on the expression of these Ribi genes is reduced in sch9 gat1 gln3 cells lacking Sch9 activity or expressing only TORC1-independent versions of Sch9 compared to cells expressing Sch9WT. It is not known why rapamycin treatment caused a stronger reduction in Ribi gene expression in the W303 compared to the TB50 genetic background. The data for TB50 cells lacking Sch9 also revealed a strong Sch9-independent component in the regulation of Ribi gene expression upon rapamycin treatment (Figure S5B).

At later time points (≥90 min), the expression of 306 genes was downregulated 3-fold in WT/WT cells. Repression of 113 of these genes was significantly (≥2-fold) dependent on Sch9. Among these, genes encoding ribosomal proteins (RPs) figured prominently. These data are consistent with previous work (Jorgensen et al., 2004), which demonstrated that both TORC1 and Sch9 regulate the expression of Ribi and RP genes.

The expression of relatively few genes was increased more than 3-fold at early time points (93), and most of these appeared to be regulated independently of Sch9. The expression of 237 genes was increased at late time points. Among these, the upregulation of 55 genes was diminished more than 2-fold in WT/2D3E cells compared to WT/WT cells. Genes regulated by Msn2/4 (http://www.yeastact.com) were concentrated in this group, suggesting that TORC1 regulates Msn2/4 activity (Beck and Hall, 1999) in part via Sch9 (Figure 6A).

Importantly, not all TORC1-regulated transcription programs appear to depend on Sch9. For example, most Gln3-regulated genes (Scherens et al., 2006), whose expression increased more than 3-fold following rapamycin treatment, did not show a significant dependence on Sch9 (Figure 6A). qPCR analyses confirmed that the rapamycin-induced expression of the Gln3/Gat1-regulated genes GLN1 and GAP1 was similar in SCH9WT, SCH9RE, and SCH92D3E cells (Figure S5C). The expression of the Rtg1/Rtg3-regulated gene CT12 was also similarly induced in sch9 gat1 gln3 cells containing different alleles of SCH9 (Figure S5D). Consistent with these results, both Gln3-13myc and Rtg1-GFP translocated normally into the nucleus after rapamycin treatment of sch9 cells expressing Sch9RE (data not shown). Thus Sch9 is not required for TORC1 to negatively regulate the activity of Gln3/Gat1 (nitrogen discrimination pathway) or Rtg1/3 (retrograde signaling pathway).

TORC1 Inhibits G0 Entry via Sch9
Both TORC1 and Sch9 prevent entry into G0 by inhibiting nuclear translocation and activation of the Rim15 kinase (Pedruzzi et al., 2003). When treated with rapamycin, SCH9WT cells contained nuclear Rim15 (Figure 6B), arrested with a G1 DNA content (Figure 6C), and exhibited G0-specific phenotypes such as accumulation of the carbon reserve glycogen (Figure 6D). These readouts were not blocked in SCH92D3E cells while cells lacking Sch9 or expressing Sch9SA constitutively localized Rim15 to the nucleus and accumulated glycogen even in the absence of rapamycin. However, the expression of G0-specific genes like GRE1 following rapamycin treatment was only moderately reduced in cells expressing Sch92D3E in our microarray experiments. These results suggest that TORC1 inhibits G0 entry in part, but not exclusively, via Sch9.

To analyze whether Sch9 phosphorylation by TORC1 is also required to complement the small cell phenotype of sch9 cells (Jorgensen et al., 2004), we measured the peak volume of sch9 gat1 gln3 cells expressing plasmid-encoded versions of Sch9. Cells expressing Sch9SA were similar in size to cells containing a control plasmid (30.0 versus 31.0 μm3) and significantly smaller than cells expressing Sch9WT (43.1 μm3). Cells expressing Sch9RE and SCH92D3E yielded a peak volume of 40.2 and 36.6 μm3, respectively (data not shown).

TORC1 Regulates Translation Initiation in Part via Sch9
In yeast, rapamycin treatment leads to a rapid decrease of translation initiation (Barbet et al., 1996) and TORC1 has been proposed to regulate translation initiation via several potential targets including elf2α phosphorylation (Cherkasova and Hinnebusch, 2003) and elf4G stability (Berest et al., 1998).

To investigate whether Sch9 is required for TORC1 to regulate translation initiation, we analyzed polysome profiles generated from mock- or rapamycin-treated SCH9WT, sch9, sch9SA, and SCH92D3E cells (Figure 7A). As expected, SCH9WT cells showed a rapid arrest of translation initiation following rapamycin treatment as indicated by a 66% decrease in the polysome to 80S monosome (P/M) ratio compared to untreated cells. In cells expressing SCH92D3E only a slight reduction in the P/M ratio occurred upon rapamycin treatment (21% decrease). Protein synthesis appeared to be already compromised in untreated sch9 and sch9SA cells as judged by the reduced polysome content and, the P/M ratio of these cells was less reduced by rapamycin treatment (19% and 46% decrease, respectively) compared to SCH9WT cells.

elf2α phosphorylation increased 4-fold in sch9 cells expressing Sch9WT upon treatment with rapamycin, but only 2.7-fold when rapamycin was added to cells expressing Sch92D3E. Furthermore, even in the absence of rapamycin,
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![Diagram](image)

**DISCUSSION**

In this study, we have confirmed and extended previous work (Jorgensen et al., 2004) by showing that Sch9 is a direct substrate for TORC1 and a major component of the TORC1 signaling pathway in S. cerevisiae. TORC1 regulates ribosome biosynthesis and thus cell-size control in large part via Sch9. Sch9 is also required for TORC1 to properly regulate entry into stationary phase and translation initiation, while other processes like the expression of Gin3/Gat1 and Rtg1/3 target genes appear to be regulated by TORC1 independently of Sch9. This is consistent with previous studies that demonstrated that TORC1 uses distinct effector pathways to regulate the expression of Ribi/RP genes versus Gin3- and Rtg1/3-dependent genes (Duvel et al., 2003). In the future it will be very important to identify and characterize Sch9 substrates.

In addition to the seven sites described here, Sch9 is phosphorylated at many more residues in its N terminus, apparently due to part to autophosphorylation (U.U. and R.L., unpublished data). It is likely that inputs from other signaling pathways are integrated with those of TORC1 and Pkn kinases to regulate Sch9 activity. Indeed, cross-talk between TORC1 and other nutrient-responsive signaling pathways appears to be a recurring theme in cell growth control (Schneper et al., 2004). Last, but not least, localization and stability of Sch9 warrant further study, in particular the ligand-binding properties of its C2 domain.

**How Is TORC1 Regulated?**

Phosphorylation of the C-terminus of Sch9 is sensitive to alterations in nutrient availability and application of various stresses. This probably reflects changes in TORC1 activity or localization, as similar results were observed with a construct that contains only the C-terminal 116 amino acids of Sch9 tethered to the vacuolar membrane. However, it is possible that changes in phosphatase activity contribute to the regulation of Sch9 phosphorylation as well. The next challenge will be to determine, at the molecular level, how growth cues regulate TORC1. An intriguing observation is the finding that at least a portion of TORC1 is active at the surface of the vacuole. The vacuole is a major nutrient reservoir in yeast, and therefore the vacuolar membrane would be an ideal location for a sensor of intracellular nutrients and for the compartmentalization of nutrient-responsive signaling pathways.

It is also noteworthy that Sch9 C-terminal phosphorylation does not always correlate with growth rate. Thus, although TORC1 activity is required for growth, factors in addition to TORC1 contribute to determine steady-state translation.

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**Figure 7. Sch9 Mediates Aspects of Translation Initiation Regulation by TORC1**

(A) Changes in polysome profiles caused by rapamycin treatment (40 min, 200 ng/ml) are reduced in cells lacking Sch9 activity or expressing a TORC1-independent version of Sch9. The positions corresponding to the 40S and 60S subunits, the 80S monosomes, and polysomal ribosomes are indicated in the profile of untreated Sch9WT cells. The ratio between polysomes and 80S monosome peaks (P/M) is indicated. (B) Cells containing Sch9ΔC10 show less rapamycin-induced (30 min, 200 ng/ml) phosphorylation of elf2α (S24) compared to Sch9WT cells, while elf2α is constitutively phosphorylated in sch9 and sch9ΔA cells. Numbers indicate the increase of the elf2α-P/elf2α ratio relative to untreated WT cells. (C) Sch9 phosphorylates the yeast S6 ortholog Rps6, but not the Ala-substituted Rps6ΔA in vitro.
growth rate. The reduction in TORC1 activity following nutrient starvation or the application of stress conditions elicits, in addition to a reduction in protein synthesis, a derepression of genes encoding proteins required for the utilization of alternative nutrient sources and stress response factors. In this way TORC1 plays an important role in allowing cells to rapidly respond to changing growth conditions. Once cells have successfully adapted their metabolism to the availability of nutrients or acquired tolerance to environmental stress, TORC1 is reactivated and growth resumes. Developmental decisions such as entry into G0 phase (Peduzzi et al., 2003) or sporulation (Colomin et al., 2003) may require a prolonged inactivation of TORC1.

Is Sch9 an S6K1 Ortholog? Many groups suggest that Sch9 is the yeast ortholog of mammalian PKB/Akt (reviewed in Sobko [2006]). However, for the following reasons we suggest that Sch9 function may be more closely related to that of mammalian S6K1. (1) Like S6K1, Sch9 activity is regulated by TORC1. In contrast, Akt is regulated by mTORC2 (Sarbassov et al., 2005). S6K1 and Sch9 also share the unusual feature of having an extended sequence beyond their homologs. S6K1 mutants lacking this domain have been found to be inappropriately activated by mTORC2 (Ali and Sabatini, 2005). (2) S6K1 and Sch9 apparently perform similar functions, most notably the regulation of translational initiation. Indeed, we have found that Sch9 is able to phosphorylate Rps6, the yeast ortholog of the mammalian RP S6, in vitro, and thus by definition is an S6 kinase.

We anticipate that further characterization of Sch9 function will not only enhance our understanding of TORC1 signaling in yeast but will also reveal additional functions of mammalian S6 kinase. For example, both TORC1 and Sch9 have been implicated in coupling nutrient excess to decreased lifespan (Fabrizio et al., 2005; Kaeberlein et al., 2005; Powers et al., 2006). This raises the interesting possibility that mTORC1 and S6K1 similarly influence longevity in mammals.

EXPERIMENTAL PROCEDURES

Strains and Plasmids Yeast strains and plasmids used in this study are listed in Tables S1 and S2. All strains except those used in Figures 4C, 6A, and 6B and Figure S4 were made prototrophic for amino acids and nucleotides by introducing pJU 450 and a URA3-containing plasmid.

Western Blot and Chemical Fragmentation Analysis PPF: 10 mM NaF, 10 mM Na2, 10 mM p-nitrophenylphosphate, 10 mM Na2PO4, and 10 mM β-glycerophosphate; Pt: 1x Rochelle protease inhibitor cocktail and 1 mM PMSF. Cultures were mixed with TCA (final concentration 6%) and put on ice for at least 5 min before cells were pelleted, washed twice with cold acetone, and dried in a speed-vac. Cell lysis was done in 100 µl of urea buffer (60 mM Tris [pH 7.5]), 5 mM EDTA, 1 M urea, 1% SDS, 1 mM PMSF, and 0.5 X PPI with glass beads in a bead beater with subsequent heating for 10 min to 65°C. For HTCC cleavage 30 µl of 0.5 M CHES (pH 10.5) and 20 µl of NTCB (7.5 mM in H2O) were added and samples incubated overnight at RT before 1 vol of 2X sample buffer (+-20 mM TCEP and 0.5 X PPI) was added. Further analysis was done by SDS-PAGE and immunoblotting using anti-HA antibody 12CA5 or anti-T750-P antisera.

TORC1 Kinase Assay Preparation of Recombinant Sch9 GST-Sch9 fusion proteins were expressed in E. coli from a pGEX-6P vector. After a 3 h induction with 0.4 mM IPTG, a clarified bacterial lysate was prepared and the fusion protein was bound to glutathione Sepharose following standard procedures. Sch9 was cleaved from the GST moiety overnight at 4°C with 24 units PreScission protease (GE Healthcare) in 300 µl PreScission cleavage buffer containing 0.01% Tween 20 following manufacturer’s instructions. The supernatant was dialyzed against (1x PBS, 20% glycerol, and 0.5% Tween 20), aliquotted, and frozen at −80°C.

Kinase Assay TORC1 was purified from RL175-2d or RL175-1b cells treated with chlavin solution or 200 nM rapamycin for 30 min. Cells grown at 30°C in YPD (250 ml per assay point) to an O.D. of ~1.0 were chilled on ice, collected by centrifugation, washed with H2O, resuspended in lysis buffer (1x PBS, 10% [v/v] glycerol, 0.5% [v/v] Tween 20, PI, and PPI), transferred to 2 ml screw-cap tubes half-filled with glass beads (0.5 mm), and disrupted in a Fast Prep machine at 4°C (Bio101; 5 x 30 s at max. speed). Crude lysates were cleared of debris with two 1000 g spins and protein concentrations normalized as necessary. Extracts were precleared over CL-4B Sepharose before 7 µl of IgG Sepharose (GE Healthcare) per assay point was added and the mix rotated for 90 min at 4°C. Beads were collected in a column, washed with cold lysis buffer, and aliquoted to 1.5 ml tubes. Kinase reactions were performed in kinase buffer (1x PBS, 20% glycerol, 0.5% Tween 20, 4 mM MgCl2, 10 mM DTT, 2 µg/ml heparin, and PI [EDTA]) in a final volume of 30 µl containing ~350 ng of recombinant Sch9. Assays were started with the addition of 100 µM ATP and 50 µCi [γ-32P]ATP, shaken for 20 min at 30°C, and terminated with the addition of 8 µl of 5x SDS-PAGE sample buffer. Samples were heated to 95°C for 5 min before being separated by SDS-PAGE, stained with Coomassie, and analyzed using a BioRad Molecular imager.

Supplemental Data Supplemental Data include five figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.molecular.org/cgi/content/ full/26/5/663/DC1/.

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Author contributions: J.U., Figures 1, 2A–2D, 3A, 4A, 4C, and 5, and Figure S3; R.L., Figures 2E and 2F and Figure 2S; A.H., Figures 3B and 7C; D.M., Figure 4B; S.L., Figure 6A and Figures 4S and 4A; A.S., Figures 6B and 6D and Figures 5B–5D; V.W., Figure 6C; O.D., Figures 7A and 7B; D.A., Table S3; and manuscript preparation, J.U. and R.L.

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**Accession Numbers**

Microarray data have been deposited at https://www.ncbi.nlm.nih.gov/geo/. They are accessible with a series accession number of GSE7050. The complete data set is also available at http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_id=509.
TORC1 controls all aspects of protein synthesis via Sch9 (2nd article)

Many distal readouts of the TORC1 pathway are known but are not completely understood at the molecular level as many signaling steps have not been described. In addition, many new readouts have probably not been identified.

To address this problem on as large a scale as possible, a novel label-free quantitative phosphoproteomic method based on liquid chromatography-mass spectrometry (LC-MS) was developed. It was used to compare the phosphoproteomes of control- or rapamycin-treated yeast cells to identify novel proteins whose phosphorylation changes upon brief exposure to the drug. By repeating the experiment in SCH9De and tap42-11 cells, the observed rapamycin-induced phosphoproteomic changes could be sorted according to their dependence on either of the two direct effectors of TORC1. This study identified known and novel targets of TORC1. Independent controls by migration shift assays in SDS-PAGE or immunopurification followed by staining with phosphospecific fluorescent dyes confirmed many of the observations in LC-MS. Some new targets were known to be involved in known TORC1-influenced functions, such as Stb3, Dot6 and Tod6 in ribosome biogenesis, while others suggested that new signaling pathways or cell processes, such as the DNA damage response, are connected to TORC1.

Subsequent study focused on Maf1 whose rapamycin-sensitive phosphorylation was shown to be dependent on Sch9. Maf1 was found to be a direct substrate for Sch9 in vitro. Seven Sch9 phosphorylation sites were mapped. Sch9 was further shown to regulate 5S rRNA and tRNA synthesis by RNA Pol III in Maf1-dependent manner. Maf1 phosphorylation by Sch9 was shown to regulate its binding to RNA Pol III, but played little role in the regulation of the polymerase. These results suggested that other Sch9 targets requiring Maf1 regardless of its phosphorylation state for RNA Pol III regulation remain to be identified. During those studies, it became evident that Sch9 was also responsible of mediating part of TORC1 signals to promote RNA Pol I recruitment to the rDNA. The exact signaling mechanism connecting Sch9 to this process was not elucidated.
Personal participation to the article: all figures and analyses except the phosphoproteomic LC-MS analysis.
Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis

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The target of rapamycin complex 1 (TORC1) is an essential multiprotein complex conserved from yeast to humans. Under favorable growth conditions, and in the absence of the macrodilide rapamycin, TORC1 is active, and influences virtually all aspects of cell growth. Although two direct effectors of yeast TORC1 have been reported (Tap42, a regulator of PPa2A phosphatases and Sch9, an AGC family kinase), the signaling pathways that couple TORC1 to its distal effectors were not well understood. To elucidate these pathways we developed and employed a quantitative, label-free mass spectrometry approach. Analyses of the rapamycin-sensitive phosphoproteomes in various genetic backgrounds revealed both documented and novel TORC1 effectors and allowed us to partition phosphorylation events between Tap42 and Sch9. Follow-up detailed characterization shows that Sch9 regulates RNA polymerases I and III, the latter via Maf1, in addition to translation initiation and the expression of ribosomal protein and ribosome biogenesis genes. This demonstrates that Sch9 is a master regulator of protein synthesis.

[Keywords: Maf1; Sch9; TOR; phosphoproteomics; rapamycin; ribosome biogenesis]

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The target of rapamycin complex 1 (TORC1) and TORC2 are large essential multiprotein assemblies structurally and functionally conserved throughout eukaryotic evolution. At their structural core are the TOR proteins, which are large Ser/Thr kinases belonging to the phosphatidylinositol kinase-like kinase family [Wullschleger et al. 2006]. It is generally believed that TORC1 is active when sufficient nutrients are present and noxious stresses are absent [DH Kim et al. 2002; Urban et al. 2007]. TORC1 also appears to monitor intracellular cues: Treatment with cycloheximide, a translation elongation inhibitor, causes a potent increase in TORC1 activity [Beugnet et al. 2003; Urban et al. 2007].

Rapamycin has been an invaluable tool to study pathways downstream from TORC1. This hydrophobic macrodilide easily crosses cell membranes to rapidly and specifically inhibit TORC1. Application of rapamycin to yeast cells demonstrated that TORC1 signals promote cell growth through both the stimulation of anabolic processes, such as protein synthesis and ribosome biogenesis, and the inhibition of catabolic processes, such as autophagy, and stress-responsive transcription programs. For a comprehensive review on TORC1 signaling in yeast, see De Virgilio and Loewith (2006).

The molecular pathways linking TORC1 to its distal readouts are presently only partially characterized. At least two direct effectors downstream from TORC1 have been described: Tap42 and Sch9 [Di Como and Arndt 1996; Jiang and Broach 1999; Urban et al. 2007]. Tap42 binds and regulates PPa2A and PPa2A-like protein phosphatases [Nanahoshi et al. 1998]. Tap42 also interacts with and is directly phosphorylated by TORC1 [Jiang and Broach 1999]. Genetic evidence clearly shows that Tap42 mediates TORC1 signals to a number of distal readouts. This is based on the observation that tap42-11, a temperature-sensitive allele of Tap42, confers semidominant resistance to rapamycin at the permissive temperature of 25°C [Di Como and Arndt 1996; Duvel et al. 2003]; Indeed, in tap42-11
cells, the activation of the transcription factors Gcn4, Gln3, Gat1, and Msn2/4, and the kinase Npr1, normally observed after inhibition of TORC1 with rapamycin, is partially or, in some cases, completely blocked [Schmidt et al. 1998; Chekrasova and Hinnebusch 2003; Duvel et al. 2003; Santhanam et al. 2004].

Sch9 is a Ser/Thr protein kinase of the AGC family. It is directly phosphorylated by TORC1 at its C terminus on at least five residues, and these phosphorylation events are required for catalytic activity [Urban et al. 2007]. Replacing some or all of these residues with acidic amino acids [SCH9<sup>55</sup> and SCH9<sup>57</sup> alleles] yields versions of Sch9 that retain activity even in the absence of upstream signals from TORC1 [Urban et al. 2007]. Sch9 mediates TORC1 signals to a number of distal readouts: Sch9 blocks the induction of genes required for entry into G<sub>0</sub> by directly phosphorylating, and thereby antagonizing, the nuclear accumulation of the Ser/Thr kinase Rim15 [Wanke et al. 2008; Wei et al. 2008]. Sch9 is critical for TORC1 to antagonize elF2a phosphorylation and thus maintain efficient translation initiation [Urban et al. 2007], and Sch9 plays important roles in the regulated expression of RNA polymerase II (Pol II)-dependent genes required for ribosome biogenesis [Jorgensen et al. 2004; Urban et al. 2007]. Except for Rim15, the substrates of Sch9 involved in these processes are not known.

Ribosome biogenesis is a highly coordinated process requiring the concerted activity of the three nuclear RNA polymerases (RNA Pol I–III) [Planta 1997; Venema and Tollervy 1999]. As it consumes a high amount of cellular energy [Warner 1999], it is not surprising that ribosome biogenesis is tightly coupled to environmental growth conditions. Much of this regulation is mediated by TORC1 [Zaragoza et al. 1998; Mayer and Grummt 2006].

RNA Pol I is dedicated to the transcription of 35S pre-rRNA, which is subsequently processed to 25S, 18S, and 5.8S rRNAs [Venema and Tollervy 1999]. Among other models, RNA Pol I was proposed to be regulated by TORC1 via recruitment of the essential initiation factor Rrm3 [Claypool et al. 2004].

RNA Pol II transcription is required for expression of ribosomal protein (RP) genes and ribosome biogenesis [ribo] genes that encode proteins required for nucleolar rRNA processing and assembly of ribosomal subunits [Jorgensen et al. 2004]. TORC1 regulates the expression of both RP and ribo genes by controlling the activities of several transcription factors. Some of this regulation is mediated by Sch9 [Jorgensen et al. 2004; Urban et al. 2007].

RNA Pol III transcribes the 5S rRNA, tRNAs, and other stable noncoding RNAs [Geiduschek and Kassavetis 2001]. TORC1 regulates RNA Pol III via its conserved repressor, Maf1 [Upadhya et al. 2002]. Under favorable growth conditions, Maf1 is highly phosphorylated and is shuttled out of the nucleus. Inactivation of TORC1 results in the rapid dephosphorylation and nuclear accumulation of Maf1 [Ojcsalska-Pham et al. 2006; Roberts et al. 2006]. TORC1 has been proposed to maintain Maf1 phosphorylation by antagonizing the activity of PP2A family phosphatases, while PKA has been proposed to be the Maf1 kinase [Moir et al. 2006, Ojcsalska-Pham et al. 2006]. Dephosphorylated Maf1 binds RNA Pol III and, consequently, blocks RNA Pol III transcription via poorly defined mechanisms [Dasai et al. 2005, Ojcsalska-Pham et al. 2006, Roberts et al. 2006].

Although many distal readouts downstream from TORC1 are known, undoubtedly more remain to be identified. Additionally, the signaling cascades that couple TORC1 to its known readouts remain incompletely understood. To better characterize these pathways we wished to define the TORC1-regulated phosphoproteome. Several liquid chromatography tandem mass spectrometry (LC-MS/MS) approaches have been developed recently to quantify protein phosphorylation [Donson and Aebersold 2006; Olsen et al. 2006]. Most prominent among these are protocols based on the differential isotopic labeling of phosphopeptides enriched from protein digests that enables relative quantification between biological samples [Zhou et al. 2001; Olsen et al. 2006]. Beyond the restricted number of biological comparisons possible, these methods suffer additional limitations: Currently, only peptide ions identified using MS/MS can be quantified, and the doubling of peptide ion signals in isotopically labeled samples makes the spectra more complex [Mueller et al. 2008]. These issues can be largely overcome by label-free LC-MS-based quantitative proteomic strategies, particularly if high mass accuracy MS is used [Rinner et al. 2007]. Thereby, every detectable phosphopeptide ion signal can be tracked across multiple LC-MS feature maps, and each tracked peptide can be quantified [Mueller et al. 2007]. As a result, the quantification process is applicable across a high number of samples.

Employing these advances, we established a novel, integrated experimental and computational pipeline for the label-free quantification of cellular phosphorylation patterns between a theoretically unlimited number of related samples. We applied this technique to compare the protein phosphorylation patterns in yeast cells upon cycloheximide or rapamycin treatment. This led to the identification of many phosphorylation sites that are presumably directly or indirectly targeted by TORC1. Repeating these assays using cells expressing TORC1-insensitive alleles of TAP42 or SCH9 we were able to accurately partition rapamycin-sensitive phosphoproteins to these two main TORC1 effector branches. These studies led to the observation that Maf1 is directly phosphorylated by Sch9, and that Sch9 regulates both Maf1 localization and binding to RNA Pol III. During the course of these experiments it became apparent that Sch9 also regulates RNA Pol I activity. Altogether this work reveals new effectors downstream from TORC1 and positions Sch9 as a central coordinator of protein synthesis.

**Results**

Sch9 and Tap42 act in parallel to mediate TORC1 signals

Two direct TORC1 effectors have been reported: Sch9 and Tap42. In order to test their functional relationship, we took advantage of the SCH9<sup>55</sup> and tap42-11 alleles.
that uncouple the encoded protein from upstream regulation by TORC1 (Di Comol and Arndt 1996, Urban et al. 2007). In the TB50 yeast background SCH9DE or tap42-11 alone conferred only very slight resistance to rapamycin but, together, the two alleles showed strong synthetic rapamycin resistance with cells still growing albeit slowly, in the presence of 200 nM rapamycin (∼20 times the minimal lethal concentration for this strain; Fig. 1A). This argues that Sch9 and Tap42 function in parallel downstream from TORC1. The observation that the growth of SCH9DE tap42-11 cells is still slowed by the presence of rapamycin suggests the existence of additional direct TORC1 substrates like Sfp1 (Lempiauinen et al. 2009). Beyond Tap42 and Sch9, the molecular events that couple TORC1 to its diverse range of downstream readouts remain largely uncharacterized.

Label-free quantitative phosphoproteomic screens

To tackle this challenge on a broad scale we employed a novel label-free quantitative phosphoproteomic approach as follows (Fig. 1B): Exponentially dividing cells were either treated with drug or mock-treated with drug vehicle. Subsequently, biochemical reactions were quenched. Proteins were extracted, enzymatically digested, and phosphopeptides were enriched using titanium dioxide-based affinity chromatography (Pinnke et al. 2004, Bodenmiller et al. 2007b). LC-MS feature maps, called phosphorylation patterns, were generated from each sample, and these phosphorylation patterns were aligned using the Superhirm algorithm (Mueller et al. 2007). Analysis of these alignments revealed phosphorylation events that were significantly regulated by a given treatment in a given genetic background.

Our specific strategy employed three different screens. First, we compared protein phosphorylation patterns of exponentially growing wild-type cells treated or mock-treated with cycloheximide, a translation elongation inhibitor that, by unknown mechanisms, hyperactivates TORC1 (Urban et al. 2007). In a second screen, protein phosphorylation patterns of wild-type cells and SCH9DE cells (SCH9DE is functionally identical to SCH9DE) were compared both with and without rapamycin treatment. For the third screen, protein phosphorylation patterns of wild-type cells and tap42-11 cells were similarly compared both with and without rapamycin treatment.

In total, 30 phosphorylation patterns were generated, containing 2256 distinct phosphopeptides mapping to 751 phosphoproteins (Supplemental Table S3, Supplemental File F1). The numbers of significantly up-regulated and down-regulated phosphopeptides in each screen are summarized in Supplemental Table S4. The summarized list of regulated phosphoproteins identified in both screens 2 and 3 and of proteins that were tested in independent phosphorylation assays is shown as Table 1. The comprehensive lists of regulated phosphopeptides are published as Supplemental Material (Supplemental File F2).

Figure 1. Label-free quantitative phosphoproteomic screens. (A) Tap42 and Sch9 act in parallel downstream from TORC1. Ten-fold serial dilutions of sch9 tap42 cells complemented with indicated alleles of SCH9 and Tap42 and made prototrophic with pAH149 were spotted onto the indicated media and incubated for 3–5 d at 25°C or 37°C. (Rap) Rapamycin. (B) Strategy for label-free quantitative phosphoproteomics. Triple arrows indicate steps performed in triplicate. (C) Venn diagram of phosphopeptides identified in both screens 2 and 3. Subsets of phosphopeptides found to be up/down-regulated by rapamycin in each screen and their overlaps are shown. The overlap of phosphopeptides predicted to be down-regulated in screen 3 and up-regulated in screen 2 is not statistically significant (P = 0.25). P-values associated with the overlaps enrichment. 1*) P < 10-12, #) P < 10-31.
Table 1. Summary of the phosphoproteomic screens

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<th>Reference</th>
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List of phosphoproteins predicted to be regulated by rapamycin in both screens and/or tested to be rapamycin-sensitive by independent assays. Proteins are sorted according to their reported functions.

\(^{a}\) Up-regulated phosphopeptides; \(^{b}\) down-regulated phosphopeptides; \(^{c}\) no regulated phosphopeptides. In parentheses, regulation observed in confirmation experiments \((\text{WB})\) migration shift assay in Western blot; \(\neg \text{PS}\) phosphorylation; \(\neg \text{WB}\) up-regulation of phosphorylation; \(\neg \text{WB}\) down-regulation of phosphorylation; \(\neg \text{WB}\) no change in phosphorylation.

\(^{d}\) Previous reports showing that the phosphorylation of the indicated proteins is regulated by rapamycin in a similar manner as in the phosphoproteomic screens.

\(^{e}\) Rps6a and Rps6b have identical protein sequences.

\(^{f}\) The peptides identified for Hrb1 and Gbp2 mapped to regions of the proteins that are identical in sequence and therefore could not be attributed to one or the other.

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Quality assessment

To assess the quality of our data we first asked whether our screens were consistent with one another, and compared the phosphorylation patterns generated from independent experiments performed as part of our different screens. The overlap between the rapamycin-sensitive phosphopeptides of screens 2 and 3 was statistically significant (Fig. 1C).

Importantly, rapamycin-sensitive phosphorylation was found in proteins known to function downstream from TORC1, including Sch8, Gln3, Gat1, Atg13, Mat1, Mks1, and Btg3 (Table 1). Thus, from our repeated independent experiments we observed consistent regulatory events, including those described in the literature. These results show the versatility of label-free quantitation, and confirm that our experimental and biological pipeline is highly reproducible.

In order to illustrate that our experimental and computational pipeline generates data of high quality, we chose to further validate the regulated phosphorylation sites (Table 1). We selected proteins from all three screens, with preference for those found regulated in two independent screens, showing opposite regulation by cycloheximide and rapamycin and/or displaying a high number of regulated phosphopeptides. HA-epitope-tagged versions of these selected proteins were expressed in yeast, and their migration in SDS-PAGE was monitored by Western blotting in the hope that the regulated phosphorylation sites would generate a migration “shift.”

Dot6, Tod6, Ksp1, Sky1, and Sb3 were predicted to become hypophosphorylated after rapamycin treatment in the phosphoproteomic screens. Consistently, rapamycin treatment resulted in a faster migration of these proteins in SDS-PAGE (Fig. 2A,B), while cycloheximide had the inverse effect. Mutagenesis of some of the predicted phosphoseresines in Dot6 and Tod6 to alanines resulted in variants whose migration was no longer altered by rapamycin treatment, indicating our protocol can accurately assign phosphorylation sites (Supplemental Fig. S1A). Ydl173w [renamed Par32] for phosphorylated after rapamycin, 32 kDa and Rph1 were predicted to become hyperphosphorylated after rapamycin treatment (although some additional sites in Par32 were predicted to be hypophosphorylated after rapamycin treatment) and Pin4 was predicted to be dephosphorylated upon cycloheximide addition. Rapamycin treatment resulted in a slower migration of these proteins, while cycloheximide had the inverse effect. Syg1 and Avt1 were predicted to become hypophosphorylated after rapamycin treatment; however, no migration shift was observed after rapamycin or cycloheximide treatment for these two proteins.

As shifts in migration could result from other post-translational modifications we wished to confirm that the shifts that we had observed were indeed the result of changes in phosphorylation. To this end we treated selected immunoprecipitated phosphoproteins with λ-phosphatase in the absence or presence of phosphatase inhibitors. In each case, phosphatase treatment converted the protein to its fastest migrating species, confirming that differential phosphorylation was responsible for the observed migration shifts (Fig. 3C). In summary, eight of the 14 proteins we tested in migration shift assays [Fig. 2A,B, data not shown] gave the expected rapamycin-induced mobility shift as predicted in our phosphoproteomic screens. Immunoprecipitation experiments and staining for phosphorylated residues showed that Ded1,
which did not show any migration shift, becomes hyperphosphorylated after rapamycin treatment as predicted [Supplemental Fig. S1B]. This suggests that at least some of the identified proteins that did not present observable migration shifts are nonetheless rapamycin-sensitive phosphoproteins.

The other objective of our phosphoproteome screens was to partition rapamycin-sensitive phosphorylation events amongst the two known TORC1 substrates, Tap42 and Sch9 [Supplemental Fig. S2; Supplemental Files F1, F2]. Again, we used SDS-PAGE migration shift assays to determine if this objective had been met. For this analysis we chose Par32 (rapamycin-induced hyperphosphorylation predicted to be mediated by Tap42), Tod6 (rapamycin-induced hypophosphorylation predicted to be mediated by Sch9 and Tap42), Dot6 (rapamycin-induced hypophosphorylation predicted to be mediated by Sch9, and Maf1 [rapamycin-induced hypophosphorylation predicted to be mediated by Sch9]. As predicted, the rapamycin-induced hyperphosphorylation of Par32 was largely blocked in tap42Δ cells [Fig. 2B]. Rapamycin-induced dephosphorylation of Tod6 and Dot6 was blocked in SCH9Δ cells and was delayed in tap42Δ cells [Tap42 dependence was preserved for Tod6 but not Dot6, which fell just under the applied cutoff for Tap42 regulation] [Fig. 2B]. Lastly, Maf1 dephosphorylation showed the predicted Sch9 dependency [Fig. 2B].

Altogether, these control experiments demonstrate that our integrated experimental and computational pipeline allowed us to identify and quantify novel rapamycin-sensitive phosphorylation sites on a systems-wide scale and to accurately partition these signaling events downstream from Tap42 and Sch9.

**Novel TORC1 targets**

We identified >100 novel TORC1-dependent phosphorylation events [Supplemental Table S4; Supplemental File F2], and we wanted to assess whether these phosphoproteins are important for TORC1 to regulate cell growth. Preliminary results suggest they are: 38 of 102 corresponding deletion strains tested gave a moderate to strong rapamycin phenotype [Supplemental Fig. S3; Supplemental Table S5]. Thus, from our data we can anticipate novel functions for TORC1 and we can begin to explain how TORC1 signals to previously established readouts.

Previous studies demonstrated a role for TORC1 in starvation-induced developmental transitions [Cutler et al. 2001]. TORC1 may mediate these transitions via Ksp1, a protein kinase that is required for filamentous growth in yeast [Bharucha et al. 2008].

Pin4 and Rph1 were shown to become hyperphosphorylated upon DNA damage (EM Kim et al. 2002; Fike et al. 2004). Curiously, we found that rapamycin induced a similar hyperphosphorylation suggesting cross-talk between TORC1 and DNA damage response pathways.

Our data suggest many new links to ribosome biogenesis. We found rapamycin-sensitive Sch9-dependent phosphorylation sites in Sch3 and Dot6/Tod6 (two homologous myb-like HTH transcription factors), and these proteins have been shown recently to function as transcriptional regulators of rti1 genes [Liko et al. 2007; Badis et al. 2008; Zhu et al. 2009]. Sky1 is a conserved Ser/Thr kinase that phosphorylates pre-mRNA splicing factors of the SR family [Siebcl et al. 1999]. Given the prevalence of introns in Rp genes, this could suggest that TORC1 also plays a more direct role in ribosome biogenesis.

We chose to focus our attention on yet another protein implicated in ribosome biogenesis regulation. Specifically, we were intrigued that Maf1, a conserved repressor of RNA Pol III, was predicted to be regulated by TORC1 via Sch9 [Fig. 2B], as this regulation was thought to occur via PP2A phosphatases [O'Farrell et al. 2006].

**Maf1 is a direct target of Sch9**

Previously, we demonstrated that Sch9 phosphorylated two serines near the C terminus of Rps6 [Urban et al. 2007]. Notably, the sequence surrounding the phosphosines now identified in Maf1 bears a striking similarity to this S6 sequence [Fig. 3A]. Maf1 was reported to be dephosphorylated after rapamycin treatment, but the mechanism by which TORC1 regulates Maf1 phosphorylation was not determined [O'Farrell et al. 2006; Roberts et al. 2006]. Although PP2A family protein phosphatases have been implicated in Maf1 dephosphorylation, we confirmed earlier reports [Willis et al. 2004; Willis and Moir 2007] that Tap42 does not play a role in rapamycin-induced Maf1 dephosphorylation [Fig. 2B].

We wished to determine if Sch9 inhibition alone could cause dephosphorylation of Maf1. In our hands, SCH9 deletion mutants grow very slowly but rapidly accumulate suppressive mutations potentially confounding conclusions derived from these strains. We therefore took advantage of a previously described analog-sensitive allele of SCH9 (sch9Δ) encoding a protein that can be specifically inhibited by the bulky ATP analog 1NM-PP1 [Jorgensen et al. 2004]. Addition of 1NM-PP1 to sch9Δ, but not wild-type cells, resulted in a rapid dephosphorylation of Maf1 [Fig. 3B].

As Maf1 was reported to be regulated by the Ras–PKA pathway and to be phosphorylated in vitro by PKA [Moir et al. 2006], we wished to further explore the relative contributions of Sch9 and PKA to Maf1 phosphorylation. PKA is encoded by three genes in yeast (TPK1, TPK2, and TPK3) and is regulated by glucose in parallel to the TOR pathway [Dechant and Peter 2008]. Deletion of the three TPK genes is lethal but can be rescued by the deletion of YAK1 [Garrett and Broach 1989]. We found that Maf1 is still phosphorylated in tpk1Δ tpk2Δ tpk3Δ yak1Δ cells, and this phosphorylation is still sensitive to rapamycin treatment [Fig. 3C]. In contrast to the results obtained with sch9Δ cells, Maf1 phosphorylation was only slightly affected by addition of 1NM-PP1 to tpk1Δ tpk2Δ tpk3Δ yak1Δ cells [Supplemental Fig. S4A,B], suggesting a minor role of PKA under these conditions. Maf1 dephosphorylation during transit through diauxic shift did not show any significant differences in tpk1Δ tpk2Δ tpk3Δ yak1Δ versus wild-type cells [Supplemental Fig. S4C]. In addition, Maf1 dephosphorylation following nitrogen starvation [Fig. 3D] was partially
Figure 3. TORC1 regulates Maf1 phosphorylation via Sch9, independently of PKA. (A) Maf1 schematic. Maf1 features including phosphorylation sites and NLSs are pictured. Serines predicted to be phosphorylated in the MS data are followed by asterisks as are the Sch9 target residues in the C terminus of Rps6. The various alanine-substituted versions of Maf1 used in the kinase assays shown in D are summarized below the scheme. (B) Sch9 inhibition leads to Maf1 dephosphorylation. (C) TORC1 regulates Maf1 phosphorylation independently of PKA. (B,C) Protein extracts were prepared from cells of the indicated genotype following treatment (15 min in C) with the indicated drugs (PP1: INM-PP1). Phosphorylation of Maf1-3HA was determined by SDS-PAGE and Western blotting. (D) Sch9 couples nitrogen-dependent signals to Maf1. Prototrophic cells of the indicated genotype were grown to exponential phase in SD, filtered, and resuspended in control [+NH₄] or in ammonium-deprived medium [-NH₄]. Samples were taken at the indicated time points and analyzed for Maf1 phosphorylation by Western blotting. (E) Sch9 phosphorylates 7 serines in Maf1 in vitro. Maf1 mutants, purified from Escherichia coli, were tested as substrates for GST-Sch9⁵⁴, purified from yeast. GST-Sch9⁵⁴ is a point mutant lacking catalytic activity and was used as a negative control. Reactions were resolved by SDS-PAGE, proteins were stained with Coomassie (CBP) and the dried gel was analyzed for ³²P incorporation.

While this manuscript was in preparation, Lee et al. [2009] reported that Sch9 phosphorylates Maf1; but, in contrast to our results, their work suggested that Sch9 and PKA perform equally important roles in Maf1 regulation. We believe that this discrepancy is due to protocol differences in the handling of yeast cells prior to protein extract. In our studies, whenever possible, TCA was added to growing cultures to quench all enzymatic activity prior to further manipulations. In contrast, Lee et al. [2009] cooled the cells on ice prior to lysis. We found that this cooling step elicits a PKA-dependent rephosphorylation of Maf1 [Supplemental Fig. S8]. This observation suggests a more prominent role of PKA in Maf1 phosphorylation at lower temperatures and explains the overestimated importance in Maf1 regulation at 30°C assigned to PKA by Lee et al. [2009].

Next, we asked if Sch9 could directly phosphorylate Maf1 in vitro. We found that Sch9⁵⁴, but not a kinase-dead mutant, was able to phosphorylate purified recombinant Maf1 [Fig. 3E]. Maf1 contains six motifs fitting the R[R/K][K][S] consensus, which is often attributed to AGC family kinases [S90, S101, S177, S179, S209, and S210]. The phosphopeptide identified in our phosphoproteomic screens [Fig. 3A] contains two overlapping copies of this motif. Unfortunately, neither the Sequen program used to annotate the tandem mass spectra nor manual inspection allowed us to determine with certainty which of the four consecutive serines in this peptide were phosphorylated. To resolve this issue we generated various alanine-substituted versions of Maf1 [Fig. 3A] and assayed the ability of Sch9 to phosphorylate these proteins in vitro. First we replaced all six serines fitting the R[R/K][K][S] consensus with alanine. This version [Maf1⁵⁴] was still phosphorylated in vitro, albeit very poorly, by Sch9 [Fig. 3E]. Based on the phosphopeptide that we identified in our screen we chose to substitute an additional serine residue [S179] generating a version of Maf1 we refer to as Maf1⁵⁴[TA]. Maf1⁵⁴[TA] was not a substrate for Sch9 in vitro, suggesting that we mapped all of the Maf1 residues phosphorylated by Sch9.

TORC1 regulates RNA Pol III through Sch9

Having established that TORC1, via Sch9, regulates Maf1 phosphorylation we next asked whether this cascade is physiologically important for the regulation of RNA Pol III function. To begin, we confirmed that rapamycin treatment causes a dramatic reduction in tRNA and 5S rRNA synthesis as determined by ³²P-huracil pulse labeling [Fig. 4A]. This drop in RNA Pol III activity was largely blocked in cells expressing SCH9⁵⁴, whereas tap42-11 alone seemed to play little if any role in this process [Fig. 4A]. These observations demonstrate that Sch9 influences RNA Pol III activity.

To extend these observations we wished to determine if Sch9 signals to RNA Pol III via Maf1. 1NM-PP1 addition to sch9⁵⁴ cells resulted in a rapid inhibition in tRNA and 5S rRNA synthesis [Fig. 4B]. MAF1 deletion abrogated the 1NM-PP1-induced reduction of tRNA synthesis but seemingly did not abrogate the reduction of 5S rRNA synthesis. An explanation for this result is explored below. Pre-tRNAs are rapidly processed in exponentially growing cells, and their abundance can thus be used to infer RNA Pol III activity. Quantitative RT–PCR analyses of the pre-tRNA⁰⁰ levels were consistent with the ³²P-huracil pulse labeling experiments and confirmed the epistasis between SCH9 and MAF1 [Supplemental Fig. S5].
These observations prompted us to determine whether the deregulation of tRNA synthesis observed in our sch9Δ maf1 mutant could lead to an altered abundance of tRNA relative to other RNA species. To this end we compared 5.8S, 5S, and tRNA levels in exponentially growing cells in the presence or absence of Sch9 and/or Maf1 function. Although total 5S tRNA levels relative to RNA Pol I-derived 5.8S tRNA were unchanged in any of the strains examined, the tRNA:5.8S rRNA ratio was approximately twofold higher in 1NM-PPI-treated sch9Δ maf1 cells compared with wild-type cells (Fig. 4C,D). An explanation for this result is explored below. To evaluate the importance of Maf1 phosphorylation in RNA Pol III regulation we employed Maf1 mutants where all seven Sch9 phosphorylated residues were replaced with either glutamate (Maf1G7) or alanine residues (Maf1A7). Inhibition of Sch9 in cells expressing Maf1G7 leads to tRNA accumulation intermediate to that observed in MAF1 and maf1 cells; cells expressing the Maf1A7 mutant had reduced basal levels of tRNA (Fig. 4C,D). [H]uracil pulse labeling experiments following Sch9 inhibition and rapamycin treatment in MAF1, maf1, MAF1Δ, and MAF1Δ
strains similarly demonstrate that Mafl phosphosite mutants are partially functional [Supplemental Fig. S6A,B]. These results are consistent with Sch9 regulating RNA Pol III via Mafl, but suggest that Mafl phosphorylation plays only a partial role in this regulation, implying that Sch9 targets additional factors to regulate RNA Pol III albeit in a Mafl-dependent manner.

Additional genetic observations further support our observations. In the presence of 1NM-PP1, sch9Δ cells grew slowly, dividing every 150 min ± 8 min [compared with 103 min ± 4 min for wild-type cells]. This slow growth rate was slightly, but significantly, improved to 135 min ± 5 min by deletion of MAFl, suggesting that reduced RNA pol III activity is one of multiple growth-limiting consequences resulting from loss of Sch9 function. Conversely, we observed a synthetic growth defect when sch9Δ mafl cells were grown at 37°C on the nonfermentable carbon source glycerol [Fig. 4E]. This result fits with the previously proposed model that accumulation of tRNA in mafl cells is detrimental to mitochondrial function [Boguta et al. 1997].

Sch9 regulates Mafl localization and association with the RNA Pol III machinery

How does phosphorylation by Sch9 alter the ability of Mafl to inhibit RNA Pol III activity? We explored two potential mechanisms by which Sch9 might regulate Mafl: Mafl localization and the capacity of Mafl to bind to RNA Pol III. Mafl was both nuclear and cytoplasmic in our strain background and prominently accumulated in the nucleus upon rapamycin treatment [Supplemental Fig. S7A,B]. Sch9Δ did not block the rapamycin-induced nuclear accumulation, but, probably due to the hypomorphic nature of these alleles [Jorgensen et al. 2004; Urban et al. 2007], SCH9Δ and sch9Δ cells showed increased basal nuclear accumulation of Mafl that could be further enhanced in sch9Δ cells by 1NM-PP1 treatment [Supplemental Fig. S7A-D]. From these results, we propose that Mafl phosphorylation by Sch9 contributes but is not sufficient to prevent Mafl nuclear localization. This hypothesis is consistent with previous models [Moir et al. 2006] suggesting that TORC1 regulates the two redundant nuclear localization sequences (NLSs) found in Mafl [Fig. 3A] independently; activity of the N-terminal NLS was proposed to be regulated via phosphorylation (i.e., Sch9-dependent) while the C-terminal NLS is regulated independently of phosphorylation (i.e., Sch9-independent).

In contrast to localization, the ability of Mafl to directly bind to RNA Pol III appears to be a more important mechanism by which Mafl phosphorylation regulates RNA Pol III activity [Osticjalska-Pham et al. 2006; Roberts et al. 2006]. Therefore we wished to test if phosphorylation by Sch9 alters the ability of Mafl to interact with the RNA Pol III subunit Rpo82. Using a coprecipitation assay we confirmed that rapamycin treatment strongly increases the association of Mafl with Rpo82. Importantly, we found that Sch9Δ blocked this rapamycin-induced interaction [Fig. 4F]. Consistent with this observation, the MaflΔ mutant did not associate with Rpo82 upon rapamycin treatment [Fig. 4F], while the MaflΔ mutant showed constitutive interaction in untreated wild-type cells [Supplemental Fig. S8A]. We were not, however, able to detect an induction of the interaction upon Sch9 inhibition in the TB60 genetic background [Supplemental Fig. S8A], which is likely due to Mafl rephosphorylation that occurs during cooling prior to nondenaturing protein extraction [Supplemental Fig. S8B]. We observed a small induction of the interaction upon Sch9 inhibition in the W303 background that could be further enhanced by concomitant inhibition of PKA [Supplemental Fig. S8C]. Taken together, these results demonstrate that Sch9 [and PKA in some conditions] regulates the capacity of Mafl to bind RNA Pol III.

Sch9 mediates the TORC1 signal to RNA Pol I

As noted above [Fig. 4C,D], we observed that the ratio of tRNA:5.8S rRNA is increased in 1NM-PP1-treated sch9Δ mafl cells relative to untreated sch9Δ mafl cells. It was not clear why tRNA should be accumulated relative to 5.8S rRNA in these exponentially growing cells, i.e., when RNA expression should be fully depressed. We rationalized that this result could be explained if both RNA Pol I and III activities are reduced upon Sch9 inhibition, with MAFl deletion suppressing only the latter. Thus, tRNA synthesis was not up-regulated per se, but rather RNA Pol I-dependent RNA synthesis was decreased in these cells. This hypothesis could also explain the apparent failure of MAFl deletion to rescue the decrease in RNA Pol III-dependent SS rRNA synthesis resulting from Sch9 inhibition [Fig. 4B]; 55 rRNA transcription, we speculate, is rescued by MAFl deletion. However, because RNA Pol I activity is reduced, 5S rRNA is produced in excess relative to other rRNAs and, consequently, is unstable and rapidly degraded. The shorter unstable RNA species whose levels increased when Sch9 activity was inhibited in mafl cells [asterisk in Fig. 4B] fits well with this notion. These deductions prompted us to test if TORC1 regulates RNA Pol I via Sch9.

We first confirmed that rapamycin treatment results in a rapid decline in the synthesis of 25S, 18S, and 5.8S rRNAs, as judged by [H]-uracil pulse labeling assays [Fig. 5A]. This effect was dramatically blocked in SCH9Δ cells but unaltered in tap42Δ cells. Interestingly, [H]-uracil incorporation into these rRNAs was virtually insensitive to rapamycin in SCH9Δ tap42Δ cells. Consistently, addition of 1NM-PP1 to sch9Δ cells resulted in a rapid, Mafl-independent, decrease in the synthesis of 25S, 18S and 5.8S rRNAs [Fig. 5B].

These results clearly demonstrated that Sch9 is indeed important in regulating the synthesis of RNA pol I-derived RNA species and beg the question: How? Sch9 could regulate RNA pol I transcription or RNA processing or a combination thereof. To begin to discriminate between these possibilities we first determined the relative amounts of unstable RNA species to try to gauge the flux from pre-rRNAs to mature end products. Rapamycin treatment and Sch9 inhibition led to decreased rRNA processing as judged by dramatically increased 27S:25S...
and 20S:18S ratios (Fig. 5A,B). Rapamycin-induced processing defects were largely blocked by the SCH9<sup>2×</sup> allele. Thus, Sch9 activity promotes rRNA processing.

We also explored if Sch9 might regulate RNA Pol I activity by several approaches. First, we used primer extension assays to determine the relative abundance of the short-lived 35S pre-rRNA. In wild-type cells, 35S levels dropped approximately threefold when assayed after 30 min of rapamycin treatment (Fig. 5C). SCH9<sup>2×</sup> and tap42-11 cells showed slight, but significant resistance, while SCH9<sup>2×</sup> tap42-11 cells showed pronounced resistance to this rapamycin-induced drop in 35S pre-rRNA levels. Consistent with these observations, addition of 1NM-PP1 to sch9<sup>2×</sup> cells resulted in an approximately twofold drop in 35S pre-rRNA levels (Fig. 5D). Although it is difficult to separate processing effects from RNA pol I
activity, these observations suggest that Sch9 and Tap42 each play a role in regulating RNA Pol I transcription.

Next, we used chromatin immunoprecipitations (ChIPs) to more directly evaluate a role for Sch9 and/or Tap42 in RNA Pol I activity. ChIPs with Rpa190, the catalytic subunit of RNA Pol I, showed that RNA Pol I occupancy at the rDNA locus decreased more than twofold following 30 min of rapamycin treatment [Fig. 5E]. Moreover, the 3H-uracil labeling assays, this rapamycin-induced eviction of RNA Pol I from the rDNA locus was strongly blocked in SCh9ΔE cells and completely blocked in SCh9ΔE tap42-11 cells. Thus, TORC1 promotes the recruitment of RNA Pol I to the rDNA locus primarily via Sch9.

Previously, Claypool et al. [2004] had proposed that TORC1 influences RNA Pol I recruitment to the rDNA locus by promoting an interaction between RNA Pol I and Rrn3, an essential initiation factor. We therefore asked if Sch9 and/or Tap42 impinge on this Rrn3–RNA Pol I interaction by pull-down of TAP-tagged Rpa190. This appears not to be the case: The rapamycin-induced dissociation of Rrn3 and RNA Pol I in SCh9ΔE tap42-11 cells was essentially indistinguishable from the dissociation observed in wild-type cells [Fig. 5F]. These data suggest that the dissociation of Rrn3 from RNA Pol I is not the primary mechanism by which TORC1 inhibition causes a reduction in rDNA transcription. We do note, however, that Rrn3 levels drop during rapamycin treatment, and that this is blocked in SCh9ΔE tap42-11 cells.

Discussion

Sch9 is a central coordinator of protein synthesis

Previous work [Upadhya et al. 2002] had shown that phosphorylation of the RNA Pol III inhibitor Maf1 is regulated downstream from TORC1 and, consistently, a Maf1 phosphopeptide was found to be down-regulated after rapamycin treatment in our phosphoproteomic screens. However, our screens further predicted that Maf1 phosphorylation is regulated by TORC1 via Sch9. Subsequent biochemical studies demonstrated that Maf1 is likely a direct substrate of Sch9 and genetic experiments demonstrated that RNA Pol III down-regulation upon Sch9 inhibition is Maf1-dependent. At the molecular level, we found that Maf1 phosphorylation by Sch9 was important for Maf1 association with RNA Pol III. However, RNA Pol III activity is still sensitive to rapamycin in cells expressing a "phosphomimetic" version of Maf1 [Maf1Δ74]. Thus, we believe that Sch9 must target a factor[s] in addition to Maf1 to regulate RNA Pol III activity.

Expectedly, MAF1 deletion, which mostly affects tRNA levels, did not suppress the sch9 growth phenotype. However, it would be interesting in the future to study the impact of Maf1 on other Sch9 phenotypes such as cell size regulation and longevity [Jørgensen et al. 2004; Kaeberlein et al. 2005]. In particular, regulation of rRNA and especially initiator tRNAΔMet levels by Maf1 could affect translation and, via Gcn4, longevity as recently observed [Steffen et al. 2008].

During the course of these studies we found that, in addition to RNA Pol III, Sch9 also regulates the synthesis of RNA Pol I transcripts. Specifically, Sch9 promotes both processing of rRNA species and recruitment of RNA Pol I to the rDNA locus. tRNA processing could be an indirect function of Sch9 as Sch9 controls the expression of many rRNA processing factors in the rib1 regulon [Jørgensen et al. 2004]. We do not know the mechanism by which Sch9 promotes recruitment of RNA Pol I to the rDNA locus but it does not appear to involve the association of RNA Pol I with its initiation factor Rrn3. Interestingly, RNA Pol I activity was proposed to be determinant for the expression of other ribosomal components (RPs and 5s rRNA), which could suggest that Sch9 regulates RP gene expression indirectly via RNA Pol I activity [Laferte et al. 2006].

Sch9 was thought previously to be the ortholog of mammalian Akt. However, we proposed recently that Sch9 functions more similarly to mammalian ribosomal S6 kinase (S6K1) [Urban et al. 2007]. This present study strengthens the functional similarities between Sch9 and S6K1: Both Sch9 and S6K1 have now been shown to regulate the activities of the three nuclear RNA polymerases (Zhang et al. 2005; Woiwode et al. 2008). Recent studies have shown that elevated RNA Pol III transcription is necessary, or in some cases sufficient, for cellular transformation [Johnson et al. 2008; Marshall et al. 2008]. Extrapolating from our results, it will be of interest to determine if dysregulation of S6K1 and/or Maf1 likewise contribute to cellular transformation.

In summary, the present study, together with previous work, demonstrates that Sch9 regulates the activities of all three nuclear RNA polymerases. In addition, we showed previously that Sch9 also regulates translation initiation [Urban et al. 2007]. Thus, Sch9 appears to play a central role in coupling environmental cues to the coordinated expression, assembly, and activity of the protein synthesis machinery [Fig. 6]. In addition to Maf1, our characterization of the rapamycin-sensitive phosphoproteome also uncovered other TORC1/Sch9 targets implicated in ribosome biogenesis, namely, Srb3 and Dot6/Tod6, which have been shown previously to respectively bind RRPE and PAC elements in rib1i gene promoters [Likó et al. 2007; Badis et al. 2008; Zhu et al. 2009]. We found Sch9-dependent rapamycin-sensitive phosphorylation sites in Srb3 and Dot6/Tod6, many of which fit the R/R/K/K/S consensus motif, suggesting that these proteins could be directly phosphorylated by Sch9. Western blot analyses confirmed that Dot6 and Tod6 are indeed phosphorylated downstream from Sch9 [Fig. 2B], and preliminary experiments suggest that Sch9 signals antagonize the ability of Srb3, Dot6, and Tod6 to inhibit rib1i gene expression [Fig. 6; Supplemental Figs. S1A, S3; data not shown].

Label-free quantitative phosphoproteomic screens

The integrated experimental and computational framework that we present in this work enables relative quantification of phosphorylation patterns. The procedure
is technically robust and sensitive, and the data acquired is accurate and, as we illustrate in this manuscript, highly reproducible. The main advantage of our method is that it allows us to quantify thousands of phosphorylation sites between, in principle, an unlimited number of samples or biological states. In addition, no a priori knowledge of phosphopeptide ions is required for quantitation as targeted LC-MS/MS methods can be employed to identify regulated ions of interest [Schmidt et al. 2008]. This improves the sensitivity and especially the achievable throughput compared with quantification based on isotope labeling.

Although our data identified many of the rapamycin-sensitive phosphorylation events described in the literature, many were missed. Indeed, high coverage of a given phosphoproteome remains a major challenge in current phosphoproteomics [Bodenmiller et al. 2007b]. Nevertheless, this study demonstrates that even with an incomplete coverage of a phosphoproteome our experimental and computational pipeline can elucidate novel and important biological phenomena as demonstrated with the characterization of Maf1 phosphorylation.

Materials and methods

Yeast cultures and assays

*Saccharomyces cerevisiae* strains and plasmids are described in Supplemental Tables S1 and S2, respectively. Strains were constructed according to standard protocols. SCH9 and TAP42 were deleted in diploid strains and complemented with plasmids encoding wild-type alleles before sporulation. Wild-type alleles were subsequently replaced with mutant alleles in haploids by plasmid shuffling.

Unless specified otherwise, rapamycin was used at 200 nM (from a 1 mM stock solution in 90% ethanol, 10% Tween-20), 1NM-PPT at 200 nM (from a 1 mM stock in DMSO), and cycloheximide at 25 μg/mL (from a 10 mg/mL stock solution in H2O).

Label-free phosphoproteomics

Cells were grown in SC—LEU 0.2% Gln at 3% to OD600 0.8 and subjected to the indicated treatments for 30 min. All biochemical activities were then quenched by the addition of trichloroacetic acid and proteins were extracted under denaturing conditions. Three 400-μL aliquots for each condition were processed separately for disulfide bond reduction, cysteine alkylation, trypsin digestion, and phosphopeptide enrichment as described in more detail in the Supplemental Material.

The phosphopeptides were separated by reverse phase chromatography on an Ekiseg-nano-LC and were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Electron Corporation) interfaced with a nano-electrospray ion source as detailed in the Supplemental Material. The LC-MS/MS data was searched against the SGD yeast protein database as described in the Supplemental Material.

For the detection of the regulated features (peak picking and integration of the area from the LC-MS data, alignment of features over multiple runs) the SuperHrm version 2.0 algorithm (Mueller et al. 2007, Runner et al. 2007) was used. Of note, as we used a label-free approach, the peptide sequence information from all LC-MS/MS runs was usable to assign the LC-MS features present in the SuperHrm output file, called MasterMap (the relevant parameters used are published as Supplemental Material). The MasterMap was post-processed as follows: Of all of the phosphopeptide features the deconvoluted masses were computed and the peak areas of phosphopeptides present in different charge states were merged. Based on these areas, the statistical significance was computed using a t-test as implemented in the Correlated software environment (Bruinsma et al. 2008).

Phosphopeptides were considered to be cycloheximide- or rapamycin-sensitive if their abundance relative to untreated cells was altered twofold or more (P-value, <0.05) by cycloheximide or rapamycin treatment, respectively. Rapamycin-sensitive phosphopeptides were considered to be Sch9-dependent after fulfilling two selection criteria. First, rapamycin-induced changes in wild-type cells had to be blunted twofold or more in *SCH9*Δ cells. Second, the abundance of a phosphopeptide reduced [or induced] by rapamycin treatment in wild-type cells had to be ≥1.5-fold higher [or lower] in both untreated and rapamycin-treated *SCH9*Δ cells compared with rapamycin-treated wild-type cells. The same criteria were used to evaluate TAP42 dependence: All regulated phosphopeptide ions corresponding to the phosphorylation sites of interest were validated.

Data availability

All MS2 information will be made available via the Phosphopep database [http://www.phosphopep.org](http://www.phosphopep.org) (Bodenmiller et al. 2007a). The raw data in the mzXML format can be downloaded from the Peptide Atlas Web page at [http://www.peptideatlas.org/repository](http://www.peptideatlas.org/repository) (Desiere et al. 2006).

Copurification assays

Precultures grown in plasmid-selective synthetic medium were diluted in YPD and grown to OD600 0.7–1.0. One-hundred-milliliter aliquots were treated as described in the text and processed for native protein extraction as described in the Supplemental Material. Protein concentrations were normalized and aliquots were removed to control for input. TAP pull-downs were performed for 2 h at 4°C with 10 μg magnetic beads [epoxy
M270 Dynabeads Invitrogen covalently coated with purified rabbit IgG. Beads were washed three times with lysis buffer before resuspension in SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and Western blotting.

\[ ^3H \]uracil labeling

Cells were grown at 25°C in SC -URA to OD\textsubscript{600} 0.6-0.9 and treated as described in the text. Five-milliliter aliquots were removed, supplemented with 50 \( \mu \)Ci 5,6-\(^{3}H\)-uracil, and incubated for 20 min. Chase was performed for 20 min with a 100-fold excess cold uracil before total RNA was extracted and analyzed as described in the Supplemental Material.

**Primer extension assays**

Primer extension assays were performed as described previously [Claypool et al. 2004] with slight modifications. The protocol is detailed in the Supplemental Material.

**Recombinant protein expression and purification-kinase assays**

Purification of GST-Sch\textsuperscript{Sch9\textsubscript{wt}} from yeast cells was performed as described previously [Urban et al. 2007], except that SCH9\textsuperscript{Sch9\textsubscript{wt}} was used as the active SCH9 allele. Many proteins were expressed in bacteria, affinity-purified using the pGEXPL system (Invitrogen), and assayed as substrates for GST-SCH9\textsuperscript{Sch9\textsubscript{wt}} as described previously [Urban et al. 2007].

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**References**


**GENES & DEVELOPMENT**

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Sch9 regulates the ribi/RP gene transcriptional repressors Stb3, Dot6 and Tod6

Ribl/RP gene regulation

Sch9 regulates the activity of all three nuclear RNA polymerases downstream of TORC1, which makes it a central node in ribosome biogenesis regulation (Huber et al., 2009a; Urban et al., 2007). Specifically, Sch9 regulates RNA Pol I recruitment to the rDNA, stimulates ribi and RP gene transcription by RNA Pol II, and promotes tRNA and 5S rRNA synthesis by RNA Pol III in a Maf1-dependent manner. Three transcription factors were recently proposed to be regulators of ribi/RP genes, Stb3, Dot6 and Tod6. Dot6 and Tod6 are paralogs which bind PAC elements in these promoters and were shown to act as repressors downstream of the TORC1-Sch9 and PKA pathways (Freckleton et al., 2009; Lippman and Broach, 2009). The role of Stb3 is less clear as it was proposed to act as an activator and a repressor of ribi genes by binding to the distal RRPE elements of their promoters (Liko et al., 2010; Liko et al., 2007). In addition to its effects on ribi genes, Stb3 was also shown to regulate RP genes transcription in response to glucose (Liko et al., 2010; Liko et al., 2007).

We chose to first probe in more detail the relationship between Sch9 and Stb3. We took advantage of the analog-sensitive sch9as allele (Jorgensen et al., 2004) which may be specifically inhibited by the bulky ATP analog 1NM-PP1. As previously reported (Jorgensen et al., 2004), 1NM-PP1 treatment has no effect in wt cells but leads to the downregulation of ribi and RP genes within 30 min in sch9as cells, which we could confirm in RT-qPCR experiments (Figure 7). We repeated the assays in cells deleted for STB3 to probe for its role downstream of Sch9. In the absence of Stb3, both ribi and RP genes were less downregulated upon Sch9 inhibition which confirms that Stb3 plays a negative role in the expression of these genes.

We then queried the relationship between Stb3 and Dot6/Tod6 downstream of Sch9. We therefore repeated the assay in cells deleted for DOT6 and TOD6 and cells deleted for all three transcription factors (Figure 7). As previously reported (Lippman and Broach, 2009), similarly to the
deletion of STB3, disruption of Dot6 and Tod6 also partially abrogated the repression of ribi and RP genes in 1NM-PP1-treated sch9** cells. In absence of all three factors, ribi and RP gene downregulation is further suppressed, which suggests that Stb3 and Dot6/Tod6 act in parallel to regulate these genes downstream of Sch9.

**Figure 7.** Stb3, Dot6 and Tod6 and the RPD3L complex regulate ribi and RP genes downstream of Sch9.

Indicated strains were grown exponentially in YPD at 30°C and treated for 30 min with either DMSO as a control or with 300 nM 1NM-PP1. Total RNA was extracted, reverse transcribed and quantitative PCRs were performed using primers specific for the genes indicated. The plotted data are means of four independent experiments ± sd.

**Stb3, Dot6 and Tod6 regulate growth and cell size**

Ribosome biogenesis regulation is linked to cell division and cell size regulation. We therefore asked whether Stb3 Dot6 and Tod6 also regulate these processes downstream of Sch9. Loss of Sch9 activity leads to slow growth and small cell phenotypes (Jorgensen et al., 2004), which are suppressed by the disruption of Stb3 or Dot6 and Tod6 (Figure 8A-C). Co-deletion of all three transcription factors further suppresses these phenotypes of SCH9, which fits with the previous observation of their parallel roles in ribi genes transcription.
Figure 8. Stb3, Dot6 and Tod6 regulate growth and cell size downstream of Sch9. A-C. \textit{SCH9} and \textit{sch9}Δ strains harboring the indicated gene deletions were grown in YPD supplemented with 300 nM 1NM-PP1 at 30 °C. A. Their steady state growth rates were measured in a microplate reader as described in the \textit{Material and methods} section. Means of three independent measurements ± sd are plotted. B-C. Steady state cell sizes were measured by FACS analysis and are plotted as a distribution of population in function of cell volume.

Stb3, Dot6 and Tod6 serve to recruit the RPD3L complex to the chromatin

We asked next how Stb3, Dot6 and Tod6 regulate \textit{ribi} and \textit{RP} gene transcription. Interestingly, all three transcription factors were found to interact physically with factors associated with the Rpd3 histone deacetylase (HDAC) which functions to repress transcription and was previously shown to inhibit \textit{ribi} and \textit{RP} gene expression (Humphrey et al., 2004; Kasten and Stillman, 1997; Rohde and Cardenas, 2003; Shevchenko et al., 2008). Indeed, the transcriptional response of \textit{Rib}i and \textit{RP} genes to Sch9 inhibition in cells deleted for \textit{RPD3} resembles the response observed in \textit{stb3 dot6 tod6} cells.
more closely than the ones of the *stb3* or *dot6* *tod6* mutants alone (Figure 7), which suggests that all
three factors might participate in recruiting Rpd3 to these promoters. In good agreement with this
hypothesis, *RDP3* deletion suppresses the growth and cell size phenotypes observed upon the loss of
*SCH9* activity to a similar degree as the co-disruption of *STB3, DOT6* and *TOD6* (Figure 8A-C).

Rpd3 acts in two distinct complexes, Rpd3S and Rpd3L, which are characterized by specific
esential subunits such as Rco1 and Sds3 respectively (Carrozza et al., 2005). Deletion of *RCO1* has
essentially no effect on *ribi* and *RP* gene regulation upon Sch9 inhibition, but disruption of *SDS3*
closely phenocopies *rpd3* mutant cells, confirming the role of the Rpd3L complex in the regulation of
these genes (Figure 7; Figure 8A-C).

Stb3 has been proposed recently to act through yet another HDAC whose deletion suppresses
the growth phenotype observed upon Stb3 overexpression (also observed in our strain background;
data not shown). However, in contrast to *STB3* deletion, deletion of *HOS2* does not suppress the *sch9*
growth phenotype (Figure 9A). Similarly, the regulation of the *ribi* and *RP* genes *DBP3, RPL9A* and
*RPS3* depends on Stb3 downstream of Sch9 but not on Hos2 (Figure 9B). These observations strongly
suggest that the SCH9-regulated activity of Stb3 in *ribi* genes repression does not require Hos2 and
that the phenotype observed upon Stb3 overexpression depends on another independent pathway
or is due to a non-physiological gain of function in Stb3 overexpressing cells.

![Figure 9](image)

**Figure 9.** The Hos2 deacetylase does not play a role in growth or ribi genes regulation downstream of
Sch9. A. Deletion of *HOS2* does not suppress the growth phenotype of *sch9* cells. *SCH9* and *sch9<sup>Δ</sup>* strains
harboring the indicated gene deletions were grown in YPD supplemented with 300 nM 1NM-PP1 at 30 °C.
Their steady state growth rates were measured in a microplate reader as described in the Material and methods section. Means of three independent measurements ± sd are plotted. B. DBP3 is regulated normally in hos2 cells upon Sch9 inhibition. Indicated strains were grown exponentially and treated with DMSO or 300 nM 1NM-PP1 for 30 min. Total RNA was extracted, reverse transcribed and the levels of the DBP3, RPL9A, RPS3 and ACT1 mRNAs were measured by qPCR. Expression of the first three were normalized to ACT1 levels and their fold regulation upon 1NM-PP1 treatment vs. DMSO treatment were plotted as means of two independent experiments ± sd.

Rpd3 was previously shown to be recruited to ribi and RP gene promoters upon TORC1 inhibition by rapamycin (Humphrey et al., 2004) and our observations suggested that Sch9 could be mediating this effect. In order to distinguish between the two Rpd3 complexes, we chose to study the physical interaction of a TAP-tagged Sds3 protein with the genome by chromatin immunoprecipitation coupled to high throughput sequencing (ChIP-Seq). The experiment was performed in cells expressing either wt or as alleles of SCH9 after 20 min of treatment with 1NM-PP1 and repeated in sch90 cells deleted for STB3 and/or DOT6 and TOD6 (Figure 10A-C). Our analysis reveals many different loci specifically enriched for Sds3, as controlled with an untagged strain (Figure 10A). A small subset of peaks was only present or was increased upon Sch9 inhibition. The inverse regulation was only rarely observed, consistent with Sch9 playing a role mainly in transcription activation. Most Sch9-dependent peaks occur upstream of RP and, to a slightly lesser extent, ribi genes. Examples are shown in Figure 10B. In the absence of Stb3 and/or Dot6 and Tod6, peaks upstream of ribi/RP genes were reduced in size or totally absent. Strikingly, enrichments upstream of RP genes, although partially dependent on DOT6 and TOD6, are usually totally absent in stb3 cells. Conversely, recruitment of RPD3L to ribi promoters appears to be heavily dependent on DOT6 and TOD6 and only partially dependent on STB3. In good agreement with this last observation, most peaks upstream of ribi genes are positioned exactly at PAC elements when one can be found in their promoters (Figure 10C). Overall, these observations strongly suggest that Stb3, Dot6 and Tod6 play a prominent role in
the recruitment of RPD3L to *ribi/RP* gene promoters. However, more careful and detailed statistical analyses should be performed to confirm these preliminary results.

**Figure 10.** The RPD3L complex is recruited at the *ribi* and *RP* gene promoters upon Sch9 inhibition in an Stb3-, Dot6- and Tod6-dependent manner. A-C. Indicated strains expressing TAP-tagged Sds3 fusion proteins (or the untagged protein as a negative control) were grown to exponential phase in YPD at 30 °C and treated for 20 min with 300 nM 1NM-PP1. Cells were then processed for ChIP-Seq analysis as described in material and methods. Sequencing read numbers are plotted on maps of the yeast genome.
W: Watson strand. C: Crick strand. A. Plot of chromosome IX. Examples of artefactual enrichments (also present in the no tag control) are indicated with red arrows. Loci enriched in an Sch9-independent manner are indicated with black arrows and loci enriched in an Sch9-dependent manner are indicated with green arrows. B. Zoom in on specific ribi (LTV1 and RRP12) and RP (RPS9A, RPL21A and RPS6A) genes. The names and positions of ORFs in these regions are indicated below the plots. C. Detailed plots of nucleotides −260 to −90 relative to the start codon of the ribi gene RRP12. RRPE and PAC elements are indicated.

**Sch9 directly phosphorylates Stb3, Dot6 and Tod6 in vitro and in vivo**

We showed previously that Stb3, Dot6 and Tod6 are phosphoproteins in vivo and become hypophosphorylated upon TORC1 inhibition by rapamycin (Huber et al., 2009a). The phosphorylation of all three proteins was predicted to be dependent on Sch9 activity which could be confirmed for Dot6 and Tod6 (Huber et al., 2009a). We therefore predicted that inhibition of Sch9 activity would lead to the dephosphorylation of Stb3, Dot6 and Tod6, which we assayed by observing their phosphorylation-induced migration shift in SDS-PAGE followed by western blotting.

Tod6 became dramatically hypophosphorylated upon Sch9 inhibition while Dot6 became only partially dephosphorylated (Figure 11A-B). This response is very similar to the one observed after rapamycin treatment (Huber et al., 2009a). Stb3 dephosphorylation only causes a mild downshift which is difficult to observe. We and others previously showed that Sch9 phosphorylates sites in motifs similar to those targeted by PKA ([R][R/K]xS*) (Huber et al., 2009a; Lee et al., 2009) and Stb3 is predicted to be phosphorylated on such motifs from our phosphoproteomic data. We therefore chose to immunoprecipitate the protein from denaturing protein extracts and probe for its phosphorylation using an antibody specifically raised against this motif. Potent dephosphorylation of Stb3 could be observed upon Sch9 inhibition using this technique, but not upon PKA inhibition (Figure 11C).

In parallel to Sch9, PKA was also shown to regulate Dot6 and Tod6 functions (Lippman and Broach, 2009). In addition, PKA was shown previously to phosphorylate Dot6 in vitro (Deminoff et al., 2006), suggesting that PKA might also regulate Dot6 and Tod6 phosphorylation in vivo. A strain
bearing analog-sensitive alleles of the three catalytic subunits of PKA (\(tpk1^{as}\) \(tpk2^{as}\) \(tpk3^{as}\)) was treated with 1NM-PP1 to compare the effects of PKA inhibition vs. Sch9 inhibition. PKA inhibition caused Tod6 to become hypophosphorylated to a similar extent as Sch9 inhibition (Figure 11B); in contrast, Dot6 was dephosphorylated in a much more pronounced manner than upon Sch9 inhibition (Figure 11A). These results correlate with the observation that PKA inhibition causes \(ribi\) genes downregulation through Dot6 more than through Tod6 while Sch9 signals equally through both transcription factors (Lippman and Broach, 2009). Inhibition of both Sch9 and PKA led to an even greater dephosphorylation of both factors compared to separate inhibition of the two kinases, which supports a model where both kinases phosphorylated Dot6 and Tod6 in parallel (Figure 11A-B).

In contrast to Dot6 and Tod6, Stb3 phosphorylation seems to be largely insensitive to PKA inhibition (Figure 11C). This observation is in agreement with previous reports suggesting that PKA is not as important as Sch9 in regulating this factor (Liko et al., 2010). However, the nature of the assay (Immunoprecipitation followed by western blotting against phosphorylated motifs) is far less reliable in detecting small changes in phosphorylation and does not detect phosphorylation at sites that are not recognized by the antibody. We therefore cannot formally rule out that PKA plays a small role in the regulation of Stb3 phosphorylation.
Figure 11. Sch9 and PKA regulate the phosphorylation of Stb3, Dot6 and Tod6 in vivo. Indicated strains were transformed with reporter plasmids bearing HA-tagged Dot6 (A), Tod6 (B) and Stb3 (C) or a control empty vector (C) and grown to exponential phase at 30°C in YPD. A-B. Cells were then treated with 1NM-PP1 for the indicated times before proteins were extracted in denaturing conditions, resolved by SDS-PAGE and blotted with anti-HA antibodies. C. Aliquots of cells were then either mock-treated with DMSO (−) or with 300 nM 1NM-PP1 (−) for 15 min before 3HA-Stb3 was immunoprecipitated from denaturing protein extracts, resolved by SDS-PAGE and probed with antibodies raised against the phosphorylated R[R/K]xS* motif or the HA epitope to control for loading. A mock IP (Left lane) was performed with the strain transformed with the empty vector to control for specificity.

We then asked whether Sch9 is capable of directly phosphorylating these three proteins. To this end, GST-tagged Stb3, Dot6 and Tod6 were expressed in *E. coli*, purified and tested as substrates in Sch9 kinase assays in the presence of radiolabeled ATP. An active Sch9 variant (Sch9<sup>3E</sup>) was able to phosphorylate the three factors, while only faint unspecific phosphorylation could be observed when
they were incubated with a kinase-dead variant of Sch9 (Figure 12). Our observations, both in vivo and in vitro, thus argue that Sch9 is a bona fide kinase for all three proteins.

**Figure 12.** Sch9 phosphorylates Stb3, Dot6 and Tod6 in vitro. A. GST alone or GST-tagged Stb3, Dot6 and Tod6 proteins were purified from E. coli and TAP-tagged Sch9 inactive (kd) and active (3E) variants were purified from yeast as described in the Material and methods section. A. Sch9<sup>3E</sup>, but not the inactive Sch9<sup>kd</sup>, phosphorylates Gst-Stb3 but not GST in in vitro kinase assays. B. Purified Sch9<sup>3E</sup> phosphorylates wt GST-Dot6 in vitro. Mutation of 5 serines to alanines in Dot6 (Dot6<sup>5A</sup>) impairs its phosphorylation by Sch9. C. Sch9<sup>3E</sup> phosphorylates wt GST-Tod6 in vitro. Mutation of 6 serines to alanines in Tod6 (Tod6<sup>6A</sup>) impairs its phosphorylation by Sch9.

**Dot6/Tod6 phosphorylation regulates their function**

We then chose to question the role of Dot6 and Tod6 phosphorylation. Dot6 and Tod6 are heavily phosphorylated proteins with more than 25 and 20 predicted phosphorylation sites respectively in large-scale mass spectrometric studies (Bodenmiller et al., 2008; Chi et al., 2007; Molina et al., 2007), probably making a systematic mapping of their phosphorylation sites extremely tedious and complicated. We therefore chose a less systematic knowledge-driven approach. By searching for R[R/K]xS* motifs in a combination of our phosphoproteomic data (Huber et al., 2009a) and large-scale phosphorylation sites mapping studies (Myers et al., 1992), we chose to mutate 5 serines in Dot6 and 6 serines in Tod6 which we felt were likely targets of Sch9 (Figure 13A). Interestingly, these sites are grouped in the middle of the proteins’ primary sequence, downstream of their N-terminal SANT-domains. As predicted, mutagenesis of these serines impaired the
phosphorylation of these proteins by Sch9 in vitro (Figure 12B-C), indicating that at least some of them are targeted by the kinase. In addition, the Sch9-activity-dependent migration shift observed in SDS-PAGE was largely lost in the Dot6 and Tod6 phosphosites mutants (both when these residues were switched to Ala or Glu; Figure 13B-C). Significant residual phosphorylation by Sch9 could still be observed in Dot6\textsuperscript{5A} and Tod6\textsuperscript{6A} mutants indicating that other sites are phosphorylated by the kinase in both proteins.

![Structure and phosphorylation of Dot6/Tod6](image)

**Figure 13.** Structure and phosphorylation of Dot6/Tod6. A. Schematic structures of Dot6 and Tod6 SANT DNA-binding domains with the positions of mutagenized phosphorylation sites indicated (| marks). B-C. Mutation of the Sch9 phosphorylation sites to alanines or glutamic acid residues in Dot6 (B) and Tod6 (C) abolishes their migration shift in SDS-PAGE. HA-tagged variants of Dot6 and Tod6 were expressed in yeast cells and proteins were extracted in denaturing conditions. Extracts were resolved by SDS-PAGE and analyzed by western blotting against the HA epitope.

We then tested the functionality of the DOT6 and TOD6 phosphosite mutants by expressing them in strains deleted for their genomic copies. We first asked whether mimicking constitutive phosphorylation of the two transcription factors would impair the regulation of their repressory activity upon Sch9\textsuperscript{e} inhibition. We chose to focus on the expression of the UTP17 mRNA whose expression is largely dependent on Dot6 and Tod6. Ectopic expression of Dot6 and Tod6 complemented partially restored UTP17 regulation by Sch9 indicating that they complemented the deletion of their endogenous copies (Figure 14). Expression of the Dot6\textsuperscript{5E} and Tod6\textsuperscript{6E} mutants complemented the deletion to the same extent. We therefore cannot argue that phosphorylation at
the mutated sites regulates the repressory activity of the two transcription factors based on this experiment.

Figure 14. Dot6 and Tod6 mutants mimicking constitutive phosphorylation are still regulated by Sch9.

Indicated strains were transformed with empty plasmids (-) or plasmids bearing wt or mutated variants of DOT6 and/or TOD6 (5E and 6E respectively). Cells were grown in plasmid-selective medium (SC –URA –LEU) at 30 ° to exponential phase. Aliquots were then treated with either DMSO or 300 nM 1NM-PP1 for 30 min before RNA was extracted and RT-qPCR assays performed to assay for the expression of the UTP17 messenger vs. ACT1. Fold regulation upon 1NM-PP1 vs. DMSO treatment were calculated and plotted as means of two independent experiments ± sd.

One possible explanation for the lack of phenotype of the glutamic acid mutants of Dot6 and Tod6 could be the redundancy of the mutated sites with other phosphorylation sites whose dephosphorylation is sufficient to activate the two transcription factors. To circumvent this problem we chose to overexpress the corresponding alanine mutants of the two transcription factors which mimic a constantly dephosphorylated state and searched for gain of function phenotypes. Overexpression of Dot6 or Tod6 shows only mild growth phenotypes in spot assays, but overexpression of their respective alanine phosphorylation sites mutants, Dot65A and Tod66A cause a
much more pronounced growth inhibition (Figure 15). In good agreement with our previous observations, the growth phenotype observed upon Dot6<sup>6A</sup> and Tod6<sup>6A</sup> overexpression was largely dependent on the presence of a functional RPD3L complex.

Figure 15. Dot6 and Tod6 phosphorylation states regulate growth. Indicated yeast strains were transformed with the indicated 2µ plasmids bearing DOT6 and TOD6 alleles under the control of a GAL1 promoter and were grown to exponential phase in SRaff –URA at 30 °C. Cells concentrations were normalized, 1/10 serial dilutions were spotted on glucose- or galactose-containing synthetic medium lacking uracil and were incubated for 2 days at 30 °C before being photographed.

Negative feedback regulation of Tod6

In various experiments, we observed that the expression levels of Tod6 were downregulated in conditions where it is dephosphorylated (Figure 16A). The Tod6<sup>6A</sup> and, to a lesser extent, the Tod6<sup>6E</sup> mutants are also expressed at lower steady state levels compared to wt (Figure 16B). Tod6 was shown to be part of the ribi regulon and a PAC element is found in its promoter (Wade et al., 2006). It is therefore likely that Tod6 regulates its own promoter in a negative feedback fashion typical of many transcription factors. To test whether this effect on Tod6 expression is transcriptional or involves destabilization of the protein or both, we measured the steady state levels of the wt, 6A and 6E alleles of the TOD6-5HA messenger RNA in parallel to the expression levels of the tagged protein by fluorescent quantitative western blotting (Figure 16B-D). Tod6<sup>6A</sup> was significantly downregulated
at both the protein and mRNA levels suggesting that at least part of the effect is due to repression of transcription. However, calculating the ratio of protein to mRNA levels shows that at least the Tod6<sup>6A</sup> mutant is further destabilized at the protein level (Figure 16E).

Figure 16. Tod6 phosphorylation regulates its own expression at the mRNA and protein levels. A. Tod6 is destabilized upon Sch9 inhibition. Indicated strains expressing HA-tagged Tod6 were grown to exponential phase in YPD at 30°C before they were treated with 300 nM 1NMPP1. Aliquots were withdrawn from the main culture at indicated time points and proteins were extracted under denaturing conditions. The Tod6-5HA and Hog1 proteins were detected by western blot analysis. B-F. The indicated Tod6 mutants were expressed in yeast cells grown exponentially in YPD at 30 °C. Data in all plots are means of three independent experiments ± sd. B. Aliquots were removed to extract proteins and assay for Tod6-5HA and Hog1 levels as in A. C. The relative expression levels of the two proteins was quantified and plotted. D. Aliquots from the same cultures were withdrawn to extract RNA. The relative expression levels of the TOD6-5HA messenger vs. ACT1 were assayed by RT-qPCR E. Expression levels of the Tod6-5HA protein relative to its mRNA were calculated and plotted. F. RNA was extracted as in D. Expression of the genomic TOD6 gene mRNA relative to the ACT1 messenger was assayed by RT-qPCR and plotted.
RNA Pol I regulation by the ribi regulon

We previously showed that Sch9 promotes RNA Pol I recruitment to the rDNA but could not determine the mechanism of regulation. The ribi regulon contains many genes involved in RNA Pol I activity, including those encoding the polymerase subunits, the initiation factor Rrn3 and the subunits of the t-UTP complex which also participated in RNA Pol I transcription initiation. It is therefore very likely that ribi gene expression levels impinge on RNA Pol I activity. It is however not clear that the transcriptional regulation of ribi genes could mediate the fast RNA Pol I response observed upon Sch9 inhibition. We therefore tested whether Stb3, Dot6 and Tod6 mediate Sch9 signals to RNA Pol I in 3H-uracil pulse labeling assays following 30 min of Sch9 inhibition at room temperature. Sch9 deactivation results in RNA Pol I downregulation and in pre-rRNA processing impairment as observed by global inhibition of 3H-uracil incorporation in 25S, 18S and 5.8S rRNA. In parallel, changes in the ratios of 27S/25S and 20S/18S 3H-uracil incorporation rates indicate that pre-rRNA processing is inhibited. Deletion of STB3 largely blocked both readouts indicating that it mediates Sch9 signals to stimulate both RNA Pol I activity and pre-rRNA processing (Figure 17). Deletion of both DOT6 and TOD6 had a lesser effect, mainly observed as a higher radioactivity incorporation rate in both the 27S and 20S pre-rRNAs in the condition of Sch9 inhibition.
Figure 17. Stb3, Dot6 and Tod6 regulate transcription initiation by RNA Pol I. A. Indicated prototroph strains were grown to exponential phase at 25°C in medium lacking uracil. $^3$H-uracil pulse chase assays were performed before and after 30 min of treatment with 300 nM 1NM-PP1. Total RNA extracts were then resolved by gel electrophoresis and transferred on positively charged membranes. Ethidium bromide staining was detected as a loading control for the displayed rRNAs and $^3$H-uracil incorporation was measured using a cyclone phosphorimager system. B-C. Indicated strains were grown to exponential phase at 30°C in YPD. Aliquots were then either mock-treated with DMSO (Ctl) or with 300 nM 1NM-PP1 for 30 min before the cells were fixed and their chromatin spread onto electron microscopy grids. Representative scans of SCH9wt and sch9ss chromatin spreads are shown (B) and polymerases numbers per rDNA repeat were quantified (C; plotted as mean ± 95% confidence interval).
To distinguish whether Stb3, Dot6 and Tod6 only play a role in the regulation of rRNA processing or also impinge on RNA Pol I activity itself, “Miller” chromatin spreads were performed, a technique which allows one to quantify the number of RNA Pol I enzymes loaded on rDNA repeats by electron microscopy providing an indirect measurement of transcription initiation rates. The number of polymerases drops by approximately 65% in sch9Δ cells following 30 min of treatment with 1NM-PP1 while it remains unaffected in wt cells, which is consistent with our previous observations (Huber et al., 2009a) (Figure 17B-C). Deletion of STB3 and/or DOT6 and TOD6 partially suppresses the downregulation of RNA Pol I initiation rates, as does the disruption of their downstream effector RPD3 (Figure 17C). These observations strongly suggest that the regulation of RNA Pol I transcription is partially dependent on the regulation of RNA Pol II transcription even on a short time scale (30 min at room temperature for pulse labeling assays).

Material and methods

Yeast strains and assays

S. cerevisiae strains and plasmids are described in Table 3 and Table 4 respectively. Strains were constructed according to standard protocols. Unless specified otherwise, rapamycin was used at 200 nM (from a 1 mM stock solution in 90% ethanol, 10% Tween-20), 1NM-PP1 at 300 nM (from 1 mM or 10 mM stocks in DMSO).

For growth assays, cells growing exponentially were diluted in the indicated media to an OD600 of 0.025 or less and 200 µl aliquots were dispensed in 96 well plates. Wells were loaded with medium without cells to serve as reference. The plates were incubated at 30 °C and OD600 was measured every 15 min for each well. Reference values were subtracted from all measurements. Linear regression was performed on the Log2 values in function of time once the cultures reached an OD600 of 0.2 and the slopes were calculated and take as a measurement of cell division per time.

Cell size assays were performed with exponentially growing cells as described previously (Jorgensen et al., 2004).
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**Table 3.** List of yeast strains used in the study

**Total RNA extraction, reverse transcription**

Total RNA extraction was performed as described previously (Laferte et al., 2006) with minor modifications. Briefly, cultures were mixed with 4 volumes of ice-cold water and were collected by
centrifugation at 2,000 x g. Cells were resuspended in 400 µl AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA) + 0.1% [w/v] SDS and lysed by shaking with glass beads in presence of 400 µl phenol solution (Sigma). Lysates were centrifuged at 21,000 x g. 350 µl of the aqueous phase was collected and extracted with 350 µl phenol-chloroform-isoamylalcohol (25:24:1; Equilibrated in AE buffer). RNA was precipitated overnight at -80°C from 300 µl of the aqueous phase mixed with 30 µl 3M sodium acetate pH 5.2 and 750 µl ethanol. RNA was pelleted by centrifugation at 21,000 x g, washed with 1 ml 80% EtOH and resuspended in H2O.

cDNA was synthesized with a mixture of random and oligo-dT primers using Bio-Rad’s iScript system. Reactions in which the enzyme was omitted were performed to control for genomic DNA contaminations.

**ChIP assays**

ChIP assays were performed and quantified by qPCR using the SYBR Green system as described previously (Bianchi et al., 2004) with slight modifications. Briefly, cells were fixed with 1 % formaldehyde for 10 min at room temperature. Fixation was stopped with 125 mM glycine and cells were harvested by centrifugation. The extracted chromatin was sheared in a bioruptor sonicator (Diagenode) for 20 min (30 s on; 30 s off) at full power. IPs were performed using pan-mouse IgG beads (Dynal) and were quantified using primers (Table 5) for the indicated loci and normalized by qPCR DNA purified from the IP input. IP efficiency was normalized with a similar quantification for the ADH4 locus (see primers above) as a control.

For ChIP-Seq experiments, ChIPs were scaled up by a factor of six (240 ml of culture at an OD600 of 0.5) and chromatin was sheared in aliquots of 300 µl for 30 min instead of 20 with otherwise identical settings. ChIPs were repeated three times (no tag controls were repeated nine times) and the immunopurified DNA was pooled, reverse crosslinked overnight at 65 °C and purified using QIAgen PCR purification kits. The DNA was eluted with 40 µl of the supplied elution buffer and stored at -80 °C until further analysis. Libraries were prepared using ChIP-Seq sample preparation kits (Illumina) according to the manufacturer’s instructions. In more details, DNA fragments ends were
repaired using a mix of klenow DNA polymerase, T4 DNA polymerase and T4 polynucleotide kinase. DNA was then purified and 3’ A overhangs were added using a klenow fragment (3’ to 5’ exo minus). DNA was purified again and ligated to adapters. 190 ± 10 bp fragments were selected using the E-Gel SizeSelect system (Invitrogen) and purified. Fragments with adapters were finally enriched with 18 cycles of PCR and purified. High throughput sequencing of libraries was performed on a Genome Analyzer IIx machine (Ilumina) each in a separate channel. Sequencing reads were mapped were mapped on SGD1.01 genome assembly using Bowtie 0.12.1 (Langmead et al., 2009) with parameters -n 3 --best --strata --solexa1.3-quals -a -m 20 and viewed with the USCS Genome Browser (Kent et al., 2002).

**Quantitative PCRs**

Quantitative PCRs were performed in 384 well plates using the Roche LightCycler 480 system. Reaction of 10 µl were prepared (5 µl 2x LightCycler 480 qPCR mix, 1 µl of primers at 2.5 µM and 4 µl template samples) according to the manufacturer instructions. RT reactions were diluted 40 fold before addition to the reaction. Primers used in qPCR reactions are listed in Table 5.
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Table 5. qPCR primers used in the study

**Denaturing protein extraction and immunoprecipitation**

Denaturing protein extracts were performed as described previously (Huber et al., 2009a).

For immunoprecipitation, denatured extracts were diluted 10 fold in native lysis buffer (PBS 10% glycerol 0.5% Tween-20) + Pi + PPi. 10 µl anti-HA beads was added and incubated for 2 hours at 4°C. Beads were washed 3x with native lysis buffer and resuspend in 10 µl protein sample buffer.

**Sch9 kinase assays**

TAP-Sch9 variants were expressed and purified as described previously (Huber et al., 2009a) except that magnetic beads coated with rabbit IgG proteins were used instead of glutathione-coated sepharose beads and that the purified kinase was not eluted from the beads.

Recombinant GST, GST-Stb3, GST-Dot6 and GST-Tod6 fusion protein variants were expressed using the pGEX6P1 system as described previously (Huber et al., 2009a) except that the proteins were eluted using PBS 20% glycerol 0.5% Tween-20 supplemented with 20 mM reduced glutathione for 15 min at room temperature.

Sch9 kinase assays were performed as described previously (Huber et al., 2009a).
Quantitative western blotting

The Li-Cor infrared fluorescent system was used for quantitative western blotting. All antibodies were incubated in PBS 0.01% Tween-20 (PBST) supplemented with 5% BSA. All washing steps were performed using PBST. After one hour blocking in PBST 5% BSA, Membranes were probed overnight with mouse anti-HA and rabbit anti-Hog1 antibodies. Membranes were washed three times with PBST and the primary antibodies were detected with anti-mouse and anti-rabbit secondary antibodies coupled to the infrared dyes IRDye800® (Rockland, PA, USA) and IRDye680® (Li-Cor, NE, USA) respectively. After 3 washes with PBST, fluorescence was detected using the Odyssey® IR imaging system (Li-Cor, NE, USA). The ImageJ software was used for quantification (Abramoff et al., 2004).

3H-uracil pulse labeling assays

3H-uracil pulse labeling assays were performed as described previously with slight modifications (Huber et al., 2009a). Briefly, cells were made prototroph and grown in SC –URA at 25 °C. 10 ml aliquots were removed and pulsed with 25 µCi 3H-uracil for 20 min. Cold uracil was added at a 100 fold molar excess and cells were grown 20 more minutes before harvest and total RNA extraction.
III. Discussion

New effectors and pathways downstream of TORC1

We identified in our phosphoproteomic studies more than 100 novel TORC1-dependent phosphorylation events in yeast cells and were able to sort them out according to their dependence on the two major effectors of TORC1, Tap42 and Sch9. The identification of these TORC1 effectors will allow us and others to characterize the signaling events linking TORC1 to known distal physiological readouts. For example, by building on the predictions of our phosphoproteomic analysis, we could demonstrate that the RNA Pol III repressor Maf1 and the ribi/RP gene repressors Stb3, Dot6 and Tod6 are direct substrates for Sch9. These observations help to explain how Sch9 regulates ribosome biogenesis and protein synthesis in general as explained in the next section.

Of greater interest, our data suggests that TORC1 regulates many additional physiological readouts of which we were not previously aware. The characterization of these novel TORC1 effectors will undoubtedly lead to many new breakthroughs in our understanding of how TORC1 coordinately regulates eukaryote cell physiology.

Various new kinases were connected to TORC1, such as Sky1 and Ksp1. Sky1 was shown to phosphorylate SR motifs in factors implicated in mRNA splicing (Siebel et al., 1999), suggesting a regulation of this process by TORC1. As introns in yeast are mostly found in genes that are highly transcribed such as the RP genes and therefore quite costly for the cells to translate, a nutrient and stress sensor like TORC1 is actually a good candidate to regulate that process. Ksp1 was previously shown to function in developmental transition in budding yeast (Bharucha et al., 2008). TORC1 has also been implicated in the regulation of that process and our data might suggest that it does so in part via Ksp1 (Cutler et al., 2001).

In addition to kinases, several factors acting in the mRNA deadenylation and subsequent decapping pathway were found in our data. Namely, Ccr4 and Not3, which act in a complex involved
in transcription regulation and mRNA deadenylation (Collart, 2003), were predicted to become phosphorylated upon rapamycin treatment. In parallel, Pat1, which acts as a deadenylation-dependent decapping factor was found to become dephosphorylated upon TORC1 inhibition by rapamycin (Bouveret et al., 2000; Tharun et al., 2000). Interestingly, deletion of any of the three factors leads to potent rapamycin sensitivity (Huber et al., 2009a). Altogether, these observations suggest that TORC1 might regulate gene expression at yet another level by controlling mRNA stability.

Another interesting connection suggested in our phosphoproteomic screen links TORC1 to DNA damage. Pin4 and Rph1 were found to become hyperphosphorylated after rapamycin treatment (Huber et al., 2009a). Interestingly, hyperphosphorylation of both proteins was first observed as a response to DNA damage (Kim et al., 2002b; Pike et al., 2004), suggesting that TORC1 might impinge on DNA damage response pathways or that it might be regulated upon genotoxic stress.

This high number of new putative targets is quite revealing of the number of unidentified pathways downstream of TORC1 that await to be described. It also underlines the importance of screening for phenotypes that are largely orthogonal to growth defects and changes in gene expression which are traditionally simpler to screen for. However, one important limitation of our experiments lies in the coverage of the phosphoproteome. Although we used whole cell extracts and stringent protein solubilization procedures, limitations inherent to the enrichment of phosphopeptides and to the LC-MS analysis allowed us to probe only about 25% of the estimated yeast phosphoproteome. It would therefore be of great interest to resume such screens to get a even more global view of the pathways downstream of TORC1. In addition to increasing the coverage, it would be very valuable to analyze the response to rapamycin in a time-dependent manner in order to determine some of the hierarchy of the signaling network. We used relatively short timepoints in our study (30 min at 23 °C, which, by following the dephosphorylation of Sch9 as a reporter for TORC1 activity, corresponds roughly to 10-15 min at 30 °C) to find phosphorylation events that were proximal to TORC1. However, given the relatively high number of targets identified,
even shorter timepoints could be studied to sort out which are the first phosphoproteins to be affected and mediate TORC1 signals to more distal targets. Later timepoints would also be interesting to study, but might be difficult to interpret because of changes in gene expression since our phosphoproteomic approach cannot distinguish between differences of protein abundance vs. differences in their phosphorylation.

**Ribosome and protein synthesis regulation by Sch9**

*Sch9, a functional ortholog of p70 S6 kinases?*

Our studies revealed striking functional similarities between Sch9 and S6K1/2 (p70S6Ks) in mammals, which belong to the same AGC family of kinases. First, both proteins were shown to be directly phosphorylated and thereby activated by TORC1 delineating a similar mode of regulation. Second, both proteins stimulate growth by regulating similar processes. Like Sch9, p70S6Ks were indeed shown to regulate translation and ribosome biogenesis by impinging on the activity of all three nuclear RNA polymerases (Proud, 2009; Woiwode et al., 2008; Zhang et al., 2005).

However, the mechanisms of ribosome biogenesis regulation by the TORC1 pathway do not seem to be completely conserved between yeasts and higher eukaryotes. In mammals, ribosome biogenesis regulation is predominantly regulated at the translational level downstream of mTORC1 (Proud, 2009); for example, the RP mRNAs usually bear short oligopyrimidine tracts in their 5’ UTR (5’-TOP) that repress their translation. mTORC1 activation alleviates this repression, specifically enhancing the translation of RPs (Meyuhas, 2000). Another important mRNA that is subjected to a similar translational control, although it is not under the control of a 5’-TOP, is the c-myc transcription factor mRNA (Gera et al., 2004). c-myc is a central promoter of cell growth and proliferation which stimulates ribosome biogenesis at various levels, enhancing RNA Pol I and III activities for example (White, 2008; Woiwode et al., 2008).

p70S6Ks were shown to play a role in 5’-TOP mRNAs regulation, although they seem to be dispensable in some contexts (Jastrzebski et al., 2007). In what might be more analogous to the
situation in yeast, p70S6Ks have also been shown to regulate ribosome biogenesis at a transcriptional level via regulating the recruitment of the SL1 complex (CF in yeast) to the rDNA (Zhang et al., 2005). Whether or not Sch9 performs similar functions in yeast is not clear.

Conversely, budding yeasts seem to regulate ribosome biogenesis predominantly at the transcriptional level. Transcription of ribi and RP regulons is controlled in a very fast and tight manner and, as we have shown, seems to account for a substantial part of RNA Pol I initiation and rRNA processing regulation downstream of Sch9. Such a tight and fast regulation of these types of genes does not seem to occur in the same way in mammals (Mayer and Grummt, 2006) which is consistent with Stb3, Dot6 and Tod6 having no obvious homologs in higher eukaryotes. Likewise, regulation of RNA Pol III also shows differences between budding yeast and higher eukaryotes. Mouse Maf1 phosphorylation was shown to be regulated normally in S6K1−/− S6K2−/− mouse embryonic fibroblasts compared to wt cells (Michels et al., 2010), while in yeast we could clearly show that Sch9 regulates Maf1 phosphorylation and controls RNA Pol III in a Maf1-dependent manner (Huber et al., 2009a). In contrast, p70S6Ks were shown to play a role by enhancing the stability of the TFIIIB initiation factor (Woiwode et al., 2008). Mechanisms of ribosome biogenesis regulation by Maf1, Stb3, Dot6 and Tod6 are discussed in more details in the next sections.

**Maf1 and RNA Pol III regulation**

**Maf1 phosphorylation**

Our phosphoproteomic screens predicted that Maf1 phosphorylation was regulated by Sch9 downstream of TORC1. TORC1 had been previously proposed to regulate Maf1 phosphorylation via the regulation of PP2A phosphatases in yeast (Oficjalska-Pham et al., 2006; Roberts et al., 2006). With both gain and loss of function experiments, we could show that Sch9 is indeed responsible for most of Maf1 phosphorylation regulation (Huber et al., 2009a). However, unlike other targets such as Tod6 whose dephosphorylation upon rapamycin treatment was completely blocked in SCH9OE cells, Maf1 is still slightly dephosphorylated in this mutant, which suggests the presence of other mechanisms of regulation, quite likely happening through the regulation of phosphatases, but in a
Tap42-independent manner. Another possible mechanism could implicate the direct phosphorylation of Maf1 by TORC1 which could be observed in vitro with yeast TORC1 and in vitro and in vivo in mammalian systems (Michels et al., 2010; Wei et al., 2009). However, TORC1 phosphorylation sites in yeast Maf1 were not mapped and it is still unclear whether this is an in vitro artifact or if this kinase-substrate relationship also occurs in living yeast cells (Wei et al., 2009).

**Regulation of Maf1 localization**

Maf1 nucleocytoplasmic shuttling was proposed to be controlled by its phosphorylation and to be a key a regulatory step for its activity (Moir et al., 2006). These first conclusions are tempered by the observation that RNA Pol III is still regulated normally in strains where Maf1 is constitutively present in the nucleus as observed, for example, in our strain background (Huber et al., 2009a; Murawski et al., 1994; Towpik et al., 2008). However, we cannot exclude that Maf1 localization plays a role in the fine-tuning of RNA Pol III regulation. Interestingly, Wei and colleagues recently observed that Maf1 penetration of the nucleolus is yet another step regulated by TORC1. This mechanism was shown to occur also in sch9 cells, but the inherent caveats of the SCH9 deletion mutant (see Phenotypic suppression of sch9 cells) combined to the negative feedback loop connecting ribosome biogenesis to TORC1 prevents any final conclusion on this issue. It could therefore be interesting to test further whether Sch9 is implicated in Maf1 subnuclear localization.

**Regulation of RNA Pol III repression by Maf1**

We and others could show that the stable interaction of Maf1 with RNA Pol III is tightly correlated with its phosphorylation state (Huber et al., 2009a; Oficjalska-Pham et al., 2006; Roberts et al., 2006). Consistently with the regulation of Maf1 phosphorylation by Sch9 and PKA, depending on the conditions, we found that the activity of both kinases is important to prevent Maf1 from binding and repressing RNA Pol III. In good agreement with those observations, we were able to show, using Maf1 mutants where all Sch9 phosphorylation sites are mutagenized to alanine residues, to mimic constitutive dephosphorylation, or glutamic acids, to mimic constitutive phosphorylation, that the former constantly binds to RNA Pol III while the latter does not form a stable complex in
conditions where TORC1 and Sch9 are inhibited. However, while Maf1 binding to the polymerase is strongly affected in these mutants and conditions, RNA Pol III shows only minor regulatory defects compared to the effects observed upon the deletion of its repressor. These unexpected results strongly suggest that TORC1 and Sch9 regulate the activity of other targets to regulate RNA Pol III. We expect that these unidentified putative effectors will still require Maf1 to downregulate RNA Pol III but in a manner independent of its phosphorylation by Sch9. We can only speculate what that target might be, but the initiation factor TFIIIB could be a good candidate as it is required for transcription from all types of RNA Pol III promoters and was shown to interact physically with Maf1 (Desai et al., 2005). In addition, two of its subunits, Brf1 and Bdp1, were found to be phosphorylated in vivo on R[R/K]xS* motifs in large-scale phosphoproteomic studies (Bodenmiller et al., 2008; Chi et al., 2007).

Stb3, Dot6 and Tod6 as repressors of ribi and RP genes

Phosphorylation and regulation by Sch9

Along with Maf1, the Stb3, Dot6 and Tod6 transcription factors which function as repressors of ribi and RP genes were predicted to be differentially phosphorylated upon rapamycin treatment in an Sch9-dependent manner. We could confirm in later experiments, that Sch9 indeed directly phosphorylates all three factors in vitro and in vivo (Figure 11; Figure 12) and that the phosphorylation sites targeted by Sch9 in Dot6 and Tod6 are important for their regulation (Figure 14; Figure 15; Figure 16). The function of the phosphorylation of Stb3 would also be very interesting to study. In particular, it will be of great interest to determine what aspects of Stb3, Dot6 and Tod6 function is affected through the direct phosphorylation by Sch9. The nucleocytoplasmic localization of Stb3 was shown to be controlled by the TORC1-Sch9 pathway in previous studies and is therefore a likely candidate (Liko et al., 2010), but its binding to the chromatin and/or its interaction with the RPD3L complex could be regulated as well. Dot6 and Tod6 regulation has not been studied yet but could also be ensured at any of the aforementioned steps.
**Stb3, a direct repressor of both ribi and RP genes?**

Stb3 was shown to have specific intrinsic affinity for RRPE elements, but lacks a clear DNA binding domain (Liko et al., 2007). It would therefore be very interesting to map its DNA binding domain in more details and conduct structural studies to determine if it belongs to a novel class of DNA-binding proteins or if, despite its lack of similarity at the sequence level, it structurally resembles other transcription factors.

Despite its first identification as a binding factor for RRPE elements, we find that Stb3 seems to play a more prominent role in the recruitment of RPD3L to the promoters of RP genes than to those of ribi genes (Figure 10B). This observation could point to a more direct role of Stb3 in RP gene transcriptional regulation than previously anticipated. This hypothesis could easily be tested by ChIP experiments at RP genes promoters showing a high dependency of RPD3L recruitment on Stb3. Our studies of ribi/RP gene expression also showed a more pronounced role of Stb3 in RP genes repression compared to ribi genes (Figure 7). In contrast, Dot6 and Tod6 showed a generally more potent effect than Stb3 on the ribi genes tested (Figure 7), which correlates with the observation that RPD3L recruitment at ribi gene promoters depends more on Dot6/Tod6 than Stb3 (Figure 10B). It is of course possible that the genes that we selected are not representative. Global transcriptional profiles could provide clearer answers in that matter. Also, a more detailed time course analysis would allow to distinguish the relative kinetics of each factors’ regulation.

If Stb3 directly regulates RP genes, where would it bind? The RRPE element is very A/T rich. It is therefore tempting to speculate that Stb3 might bind to the orphan A/T-rich regions found in RP gene promoters. The clearest way to test this hypothesis would be to perform Stb3 ChIP-Seq experiments, but a deeper analysis of Sds3 binding, which is strongly dependent on Stb3 upstream of RP genes, could already give us a hint about the element targeted in those promoters.

The RRPE element was also shown to mediate the regulatory activity of Sfp1 to the ribi genes (Fingerman et al., 2003). In addition, Sfp1 was shown to have intrinsic affinity for an RRPE-like motif in vitro (Zhu et al., 2009). It will therefore be interesting to investigate the functional relationship of
Stb3 and Sfp1 in regulating transcription. In particular, should the two proteins target similar elements, it would definitely be worth to check whether they compete with each other for binding upstream of their target genes.

In our studies of ribi/RP gene regulation, we find that the deletion of the Stb3, Dot6 and Tod6 transcription factors and disruption of the RPD3L complex do not completely suppress the repression caused by Sch9 inhibition (Figure 7). This result suggests that Sch9 can signal via alternative pathways to regulate these genes. We can only speculate on what they are, but it is possible that Sch9 influences the activity of other known factors binding to these gene promoters to influence, for example, the recruitment of histone acetyltransferases - such as NuA4 whose recruitment was shown to be rapamycin-sensitive (Rohde and Cardenas, 2003). Alternatively, Sch9 could influence the stability of these transcripts via yet unidentified pathways.

**The role of ribi and RP genes in rRNA transcription and processing**

The regulation of RNA Pol I downstream of TORC1 and Sch9 is still poorly understood. We showed here that regulating the transcription of ribi and RP genes has fast and significant consequences on RNA Pol I initiation rates and rRNA processing (Figure 17). Stb3 and Dot6/Tod6 do not have the same effects on both processes, Dot6/Tod6 regulating mainly transcription initiation and Stb3 repressing both initiation and processing. These differences might arise from the more pronounced role of Dot6/Tod6 in regulating ribi genes while Stb3 rather functions to downregulate RP genes.

Disruption of Rpd3 mimics quite well the rRNA transcription and processing phenotypes of stb3 dot6 tod6 cells upon Sch9 inhibition (Figure 17). This observation is in good agreement with our model of RPD3L recruitment by all three transcription factors. However, previous reports clearly showed that rpd3 cells downregulate RNA Pol I transcription upon rapamycin treatment like wt (Oakes et al., 2006). As we have shown that Sch9 plays a significant role downstream of TORC1, our data would actually predict that Rpd3 should have an effect there as well. This discrepancy could be explained in two ways. First, the deletion of RPD3 does not totally abolish RNA Pol I regulation upon
Sch9 inhibition which suggests the existence of an alternative pathway. This other pathway could account for the bypass activity observed in SCH9DE cells upon rapamycin treatment. In this model, the transcription of *ribi* and *RP* genes does not play any role in RNA Pol I regulation downstream of TORC1. Alternatively, besides its role as a repressor of *ribi* and *RP* genes, Rpd3 could also function as an enhancer of RNA Pol I transcription upon rapamycin treatment, thereby masking its first function. In this model, Sch9 would only regulate the repressory activity of Rpd3 and *ribi/RP* genes regulation would still play a role downstream of TORC1.

**Overlap of Sch9 and PKA signaling**

As shown in Figure 11, PKA regulates Dot6 and Tod6 phosphorylation in parallel to Sch9 *in vivo*. In addition, Dot6 was previously shown to be directly phosphorylated by PKA *in vitro* (Budovskaya et al., 2005), which strongly suggests that Dot6 and probably Tod6 as well are direct substrates of PKA. Dot6 and Tod6 should therefore be added on the list of the common targets of Sch9 and PKA, which already contained Rim15, and Maf1 (Moir et al., 2006; Reinders et al., 1998; Wanke et al., 2008). Interestingly, all these factors are negative regulators of growth that are inhibited by Sch9 and PKA. This signaling architecture probably provides a functional explanation to why hyperactivation or overexpression of Sch9 and PKA suppress so well the loss of each other’s function.

**Model of ribosome biogenesis and regulation by Sch9**

All of our observations can be accommodated in a model of ribosome biogenesis regulation by Sch9 where Dot6 and Tod6 strongly regulate *ribi* genes transcription by directly binding to their promoters while Stb3 has a more prominent effect on *RP* genes (Figure 18). Regulation of *ribi* genes by Dot6/Tod6 could then have an indirect effect on *RP* genes via crosstalk mechanisms which still remain to be elucidated. *Ribi* and *RP* gene transcriptional regulation in turn modulates rRNA production, both at the transcription initiation step by RNA Pol I and at the processing steps. In parallel to that Stb3-/Dot6-/Tod6-/RPD3L-dependent pathway, Sch9 also regulates RNA Pol I via pathways that remain to be elucidated.
Lastly, Sch9 promotes 5S rRNA and tRNA synthesis by inhibiting the RNA Pol III repressor Maf1. Sch9 acts at multiple steps by directly phosphorylating Maf1 and via a still unidentified target which then prevents Maf1 from repressing transcription. PKA also impinges on Maf1, Dot6 and Tod6 phosphorylation to various extents.

**Figure 18.** Model of ribosome biogenesis and protein synthesis regulation by Sch9. Arrows and T-shaped connections indicate positive and negative regulatory events respectively. Dashed connections indicate indirect regulatory mechanisms.
Abbreviations

ABD  Adaptor Binding Domain
C2   C2 lipid-binding domain
ChIP Chromatin ImmunoPrecipitation
ChIP-Seq Chromatin ImmunoPrecipitation – Sequencing
CHX  CycloHeximide
DNA  DeoxyriboNucleic Acid
eIF4G eukaryotic Initiation Factor 4G
ETS  External Transcribed Spacer
FAT  FRAP, ATM, T RRAP
FATC FAT C-terminus
FKBP12 FK506-Binding Protein 12 kDa
HDAC Histone DeAcetylation
HEAT Huntingtin, Elongation factor 3, Alpha regulatory subunit of type 2A protein phosphatase, TOR
ITS  Internal Transcribed Spacer
LC   Liquid Chromatography
MS   Mass Spectrometry
mTOR mammalian Target Of Rapamycin
NDP Nitrogen Discrimination Pathway
ORF  Open Reading Frame
PCR  Polymerase Chain Reaction
PIKK Phosphatidylinositol Kinase-like Kinase
PP1  1NM-P P1
PRD  PIKK Regulatory Domain
qPCR quantitative PCR
RBD  RAS Binding Domain
rDNA ribosomal DNA
ribi ribosome biogenesis
RNA RiboNucleic Acid
RNA Pol I-III RNA Polymerase I-III
RP Ribosomal Protein
rRNA ribosomal RNA
SANT SWI3, ADA2, N-CoR, TFIIB
sd standard deviation
snoRNA small nucleolar RNA
snRNA small nuclear RNA
TOR Target Of Rapamycin
TORC TOR Complex
tRNA transfer RNA
References


