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Abstract
Ribosome biogenesis drives cell growth, and the large transcriptional output underlying this process is tightly regulated. The Target of Rapamycin (TOR) kinase is part of a highly conserved signaling pathway linking nutritional and stress signals to regulation of ribosomal protein (RP) and ribosome biogenesis (Ribi) gene transcription. In Saccharomyces cerevisiae, one of the downstream effectors of TOR is Sfp1, a transcriptional activator that regulates both RP and Ribi genes. Here, we report that Sfp1 interacts directly with TOR complex 1 (TORC1) in a rapamycin-regulated manner, and that phosphorylation of Sfp1 by this kinase complex regulates its function. Sfp1, in turn, negatively regulates TORC1 phosphorylation of Sch9, another key TORC1 target that acts in parallel with Sfp1, revealing a feedback mechanism controlling the activity of these proteins. Finally, we show that the Sfp1-interacting protein Mrs6, a Rab escort protein involved in membrane trafficking, regulates both Sfp1 nuclear localization and TORC1 signaling.

Reference

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Sfp1 Interaction with TORC1 and Mrs6 Reveals Feedback Regulation on TOR Signaling

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SUMMARY

Ribosome biogenesis drives cell growth, and the large transcriptional output underlying this process is tightly regulated. The Target of Rapamycin (TOR) kinase is part of a highly conserved signaling pathway linking nutritional and stress signals to regulation of ribosomal protein (RP) and ribosome biogenesis (Ribi) gene transcription. In Saccharomyces cerevisiae, one of the downstream effectors of TOR is Sfp1, a transcriptional activator that regulates both RP and Ribi genes. Here, we report that Sfp1 interacts directly with TOR complex 1 (TORC1) in a rapamycin-regulated manner, and that phosphorylation of Sfp1 by this kinase complex regulates its function. Sfp1, in tum, negatively regulates TORC1 phosphorylation of Sch9, another key TORC1 target that acts in parallel with Sfp1, revealing a feedback mechanism controlling the activity of these proteins. Finally, we show that the Sfp1-interacting protein Mrs6, a Rab escort protein involved in membrane trafficking, regulates both Sfp1 nuclear localization and TORC1 signaling.

INTRODUCTION

In rapidly growing yeast cells, ribosome biogenesis is a major energy-consuming process that accounts for a significant fraction of total transcriptional output. For example, under optimal growth conditions, it is estimated that > 50% of RNA Polymerase II initiation events occur at ribosomal protein (RP) genes, or at other genes whose products are involved in ribosome biogenesis (Ribi genes) or translation (reviewed in Martin et al., 2006; Rudra and Warner, 2004; Warner, 1999). Regulation of ribosome biogenesis at the transcriptional level thus plays a key role in cellular energy homeostasis.

Regulation of RP and Ribi gene expression has been best studied in Saccharomyces cerevisiae, where several proteins have been implicated in RP gene activation, including Rap1, Ifh1, Fhl1, Hmo1, Sch9, and Sfp1 (Hall et al., 2006; Jorgensen et al., 2002, 2004; Lieb et al., 2001; Marion et al., 2004; Martin et al., 2004; Schawalder et al., 2004; Wade et al., 2004). Among these factors, the AGC kinase Sch9, a putative ortholog of mammalian S6K1 (Urban et al., 2007), and the zinc (Zn)-finger protein Sfp1 also appear to be intimately involved in the activation of Ribi genes, with epistasis analysis suggesting that the two proteins act in parallel (Jorgensen et al., 2004).

Sfp1 function is regulated, at least in part, at the level of subcellular localization. Activity of the Target of Rapamycin Complex 1 (TORC1) and Protein Kinase A (PKA) kinases, effectors of two highly conserved nutrient-sensitive growth signaling pathways in yeast, promotes Sfp1 nuclear localization (Jorgensen et al., 2004; Marion et al., 2004). Interestingly, Sfp1 appears to play a key role in coupling cell growth and division, since sfp1Δ cells are smaller than any other single-gene deletion mutant (Jorgensen et al., 2002). TOR is a large, evolutionary conserved Ser/Thr protein kinase that belongs to the family of phosphatidylinositol kinase-related kinases (PIKKs) (Wullschleger et al., 2006). TOR functions in two different multiprotein complexes (TORC1/2), each of which regulates different aspects of growth (Loewith et al., 2002). Rapamycin-sensitive TORC1 of S. cerevisiae is composed of Kog1, Tco89, Lst8, and either Tor1 or Tor2 kinase (Loewith et al., 2002; Reinke et al., 2004). Its major function appears to be the regulation of translation capacity in response to environmental signals by promoting ribosome biogenesis, amino acid availability, and translation efficiency. This is in part mediated by direct phosphorylation and activation of Sch9 by TORC1 in a nutrient- and stress-sensitive manner (Urban et al., 2007). Another important mechanism of TORC1 signaling is the regulation of PP2A-type phosphatases via Tap42 (Duvel et al., 2003).

The localization of TORC1 in yeast cells is currently under debate (Martin et al., 2006). Several earlier studies have shown that TORC1 components are associated with internal membranes (reviewed in De Virgilio and Loewith, 2006), and recent studies demonstrate that Tor1-GFP appears to be concentrated on the vacuolar membrane (Sturgill et al., 2008), where TORC1 is able to phosphorylate Sch9 (Urban et al., 2007). However, another report showed that under favorable growth conditions at least a fraction of yeast Tor1 localizes to the nucleus, but is rapidly exported to the cytoplasm after rapamycin treatment or nutrient deprivation (Li et al., 2006). Mammalian TOR (mTOR) has also been reported to shuttle between the nucleus and cytoplasm (Kim and Chen, 2000).
Here, we report that Sfp1 interacts directly with TORC1 and is phosphorylated at multiple residues, both in vivo and in vitro, in a TORC1-dependent manner. We also show that Sfp1 negatively regulates the activity of TORC1 kinase toward Sch9, suggesting the existence of a feedback loop controlling RP and Ribi gene transcription. Furthermore, we provide evidence that the Sfp1-interacting protein Mrs6, an essential Rab escort protein (Benito-Moreno et al., 1994; Fujimura et al., 1994), regulates both Sfp1 nuclear localization and TORC1 activity, thereby indicating direct links between intracellular vesicular trafficking, TORC1 signaling, and ribosome biogenesis.

RESULTS

Purification and Identification of Sfp1-Interacting Proteins

Sfp1 is a transcriptional activator implicated in the TORC1-dependent regulation of both RP and Ribi gene transcription in S. cerevisiae (Jorgensen et al., 2002, 2004; Marion et al., 2004). However, several aspects of Sfp1 function remain obscure. For example, the mechanism by which Sfp1 regulates its target genes is unclear. Although induction of Sfp1 overexpression leads to more rapid upregulation of Ribi genes than RP genes (Jorgensen et al., 2004), Sfp1 binding in vivo is only detected at RP gene promoters (Marion et al., 2004). Furthermore, it is currently unknown how TORC1 regulates Sfp1. To gain insight into these questions, we partially purified a fully functional Sfp1-TAP fusion protein, expressed from the endogenous SFP1 locus, and identified in vivo interacting proteins by mass spectrometry. Proteins identified by this approach are shown in Figure 1A (and in Figures S1A and S1B, available online) and are listed in Figure 1B (for a complete list, see Table S3).

Sfp1 Interacts with TORC1 Components

We first focused our attention on Tor1 and Kog1, since both proteins are subunits of TORC1 (Loewith et al., 2002). We confirmed the interaction between Sfp1 and TORC1 components by showing that HA-tagged versions of Tor1 and Kog1 were specifically coimmunoprecipitated with Sfp1-TAP (Figure 2A). We found that both interactions were strongly reduced when cells were treated with rapamycin prior to protein extraction (Figure 2A). Lst8, another TORC1 component (Loewith et al., 2002), also interacts with Sfp1 in a rapamycin-regulated manner (Figure 2B). In further support of the Sfp1-TORC1 interaction, we showed that both Kog1-TAP and Tco89-TAP (Tco89 is an additional component of TORC1 [Reinke et al., 2004]) can coimmunoprecipitate Sfp1-HA from cell extracts (Figure 2C). By comparing the amounts of input to immunoprecipitate, we can roughly estimate that ~10% of the immunoprecipitated Sfp1 molecules are bound by Tor1 and Kog1, and that ~5% of the TORC1 complex recovered in our extracts is bound by Sfp1.

Sfp1 Is Phosphorylated In Vivo in a TORC1-Dependent Manner

To address the possibility that TORC1 regulates Sfp1 phosphorylation in vivo, we used an in-gel stain (Pro-Q Diamond) to measure Sfp1 phosphorylation. The complete loss of the signal after phosphatase treatment shows that the stain recognizes the phosphorylated form(s) of Sfp1 (Figures 3A and S2A). The TORC1 inhibitor rapamycin caused a partial (~60%) dephosphorylation of Sfp1, which was observed 10 min after rapamycin addition and persisted for at least 1 hr. The effect of rapamycin on Sfp1 phosphorylation correlated well with its effect on the Sfp1-TORC1 interaction (Figure 3A, 3HA-Tor1). Cycloheximide (CHX) has been recently shown to increase TORC1 activity toward a different substrate, the Sch9 kinase (Urban et al., 2007). We also detected increased phosphorylation of Sfp1 after CHX treatment, which was associated with a simultaneous increase in the Sfp1-TORC1 interaction.

Wortmannin and caffeine are known inhibitors of PIKKs (Powis et al., 1994; Sarkaria et al., 1999), and caffeine has been shown to inhibit TORC1 directly (Reinke et al., 2004). We found that both wortmannin and caffeine treatments caused a dephosphorylation of Sfp1 in vivo very similar to that observed after rapamycin treatment (Figures 3B and S2B). Interestingly, these compounds had different effects on the Sfp1-TORC1 interaction; caffeine inhibited the interaction, although the effect

Table 1: Purification and Identification of Sfp1 In Vivo Interacting Proteins

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<tr>
<th>Protein(s) Identified</th>
<th>Score</th>
<th>Molecular Mass (kDa)</th>
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<td>5230</td>
<td>48 kDa</td>
<td>Rab escort protein</td>
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<td>Sfp1-TAP</td>
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<td>635</td>
<td>211 kDa</td>
<td>Transcriptional regulator</td>
</tr>
<tr>
<td>Kog1</td>
<td>414</td>
<td>179 kDa</td>
<td>Subunit of TORC1</td>
</tr>
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</table>

Figure 1. Purification and Identification of Sfp1 In Vivo Interacting Proteins

(A) SDS-PAGE of Sfp1-TAP-associated proteins, stained with silver; mock purification from untagged cells was performed as control. Protein bands identified by mass spectrometry are indicated.

(B) Table of Sfp1-interacting proteins identified. Score values, a measure of the significance of protein identifications, were derived by using Mascot software (see the Supplemental Data).
TORC1 regulates the localization of Sfp1, since rapamycin treatment results in a significant decrease in the nuclear concentration of Sfp1 (Figure 3C) (Jorgensen et al., 2004; Marion et al., 2004). As expected, both caffeine and wortmannin also reduced Sfp1 amounts in the nucleus, as did nutrient withdrawal and osmotic shock (Figures 3C and S2F). Interestingly, in CHX-treated cells, Sfp1 is even more concentrated in the nucleus than in untreated cells (Figure 3C). Taken together, these data indicate that the TORC1-Sfp1 interaction and TORC1-mediated phosphorylation of Sfp1 contribute to but are not sufficient for Sfp1 nuclear localization.

TORC1 Kinase Directly Phosphorylates Sfp1 at Multiple Sites

The results described above show that Sfp1 interacts directly with TORC1 and suggest that it may be a direct target of TORC1 kinase. To test if Sfp1 can be phosphorylated by TORC1, we performed an in vitro kinase assay. Purified Sfp1-TAP was phosphorylated in this assay, and the phosphorylation was strongly inhibited by the addition of rapamycin plus its receptor, FKBP12 (encoded by FPR1 gene), to the in vitro assay mix (Figure 4A). Both caffeine and wortmannin strongly inhibited the in vitro phosphorylation of Sfp1, whereas a casein kinase 2 (CKII) inhibitor did not. We also tested the idea that Sch9, an AGC kinase that is directly activated by TORC1 phosphorylation, might target Sfp1. However, the addition of purified Sch9 to the kinase reaction had no effect on Sfp1 phosphorylation (Figure S3A). Furthermore, neither deletion of SCH9 nor expression of a TORC1-independent “phosphomimetic” mutant of Sch9 (SCH9-3E) (Urban et al., 2007) affected the localization (Figure S3B) or phosphorylation (Figure S3C) of Sfp1 in vivo. Our in vivo and in vitro phosphorylation data thus strongly suggest that Sfp1 is a direct substrate for TORC1 kinase.

To identify potential TORC1 phosphorylation sites in Sfp1, we purified Sfp1 from logarithmically growing cells and mapped phosphorylated residues by mass spectrometry. Our analysis covered ~81% of the Sfp1 sequence (Figure S4) and identified seven potential phosphorylation sites (Figure 4B; Table S4). Mutation of these seven sites to alanine, to generate the sfp1-1 allele, caused a strong decrease in the in vivo phosphorylation status of Sfp1 (Figure 4C; compare lanes 1 and 4), indicating that at least some of the mutated residues are indeed phosphorylated in vivo in the wild-type protein. The phosphorylation status of sfp1-1 decreased only slightly (~10%) after rapamycin treatment, suggesting that most (or all) of the residues affected by TORC1 in vivo have been mutated in this allele.

Results of in vitro phosphorylation experiments were largely consistent with the in vivo data. Thus, phosphorylation of the mutant was reduced to < 50% of the wild-type phosphorylation level in untreated samples (Figure 4D). However, rapamycin treatment of the cells prior to protein purification had a clear effect on sfp1-1 in vitro phosphorylation, which was not observed in vivo. At present, we do not know whether this is an indication that additional (one or two) TORC1 target sites in Sfp1 have yet to be identified, or whether the in vitro reaction leads to phosphorylation of sites that are not targeted in vivo (or both). Finally, we note that the sfp1-1 mutation had little or no effect on the interaction between Sfp1 and TORC1 (Figure S5A).

Figure 2. Sfp1 Interacts with TORC1 Components In Vivo

(A) Tor1 and Kog1 interact with Sfp1 in a rapamycin-sensitive manner. Lysates from cells expressing 3HA-Tor1 or Kog1-3HA alone, or together with Sfp1-TAP, were subject to a single-step TAP purification. For indicated samples, rapamycin (200 ng/ml) was added 30 min prior to cell harvesting. Proteins were separated by SDS-PAGE and detected by immunoblotting with the indicated antibodies. Act1 served as control.

(B) Lst8 interacts with Sfp1 in a rapamycin-sensitive manner. Lysates from cells expressing Lst8-3HA alone or together with Sfp1-TAP were analyzed as in (A).

(C) Sfp1 associates with Kog1 and Tco89. Lysates from cells expressing Lst8-3HA alone or together with Sfp1-TAP were analyzed as in (A).

was reduced compared to rapamycin, whereas wortmannin had little or no effect. Our results suggest that rapamycin and caffeine regulate TORC1 signaling in vivo, at least in part, on the level of substrate recognition/binding, in agreement with a recent study (Yan et al., 2006).

Nutrient withdrawal causes dephosphorylation of the TORC1 substrate Sch9 (Urban et al., 2007). Interestingly, neither Sfp1 phosphorylation nor its physical interaction with TORC1 was reduced by removal of glucose from the medium (Figure S2C) or by complete nutrient withdrawal (cells shifted to H2O) (Figure 3B). Furthermore, neither growing cultures through the diauxic shift (Figure S2D) nor an osmotic shock (Figure S2E) significantly reduced either Sfp1 phosphorylation or its interaction with TORC1. Despite these differences, we note that rapamycin, caffeine, wortmannin, and CHX treatments have very similar effects on TORC1-mediated phosphorylation of Sfp1 and Sch9 (Figures 3A and 3B) (Urban et al., 2007; Wanke et al., 2008).
TORC1 Phosphorylation of Sfp1 Regulates Nuclear Localization and RP Promoter Binding

To test if TORC1-dependent phosphorylation regulates Sfp1 function, we examined the localization of sfp1-1 protein (Figure 5A). Sfp1-1 showed reduced nuclear localization compared to the wild-type protein. Consistent with the localization data, sfp1-1 binding to a RP gene promoter was also reduced compared to the wild-type (Figure 5B). Rapamycin still...
had a small effect on both sfp1-1 localization and RP promoter binding (Figures 5A and 5B), suggesting that sfp1-1 may still contain additional TORC1 target sites. Surprisingly, sfp1-1 cells displayed a normal growth rate (Figures 5C and S5C) and cell size (Figure S5B), indicating that the sites mutated do not have a major impact on either growth or size regulation in otherwise wild-type cells. However, sfp1-1 cells showed a decrease in CHX and rapamycin resistance (Figures 5D and S5D). Furthermore, when we combined the sfp1-1 mutation with a SCH9 deletion (sch9D), we observed that sfp1-1 greatly reduced both growth rate, cell size, and resistance to both CHX and rapamycin (Figures 5C, 5D, and S5B–S5D), indicating an important role for these residues that is redundant with Sch9 function, consistent with the earlier observation that sfp1D and sch9D display synthetic lethality (Jorgensen et al., 2004).

Sfp1 Regulates TORC1 Phosphorylation of Sch9

The in vivo interaction between Sfp1 and TORC1, together with the genetic interaction between SFP1 and SCH9, prompted us to test whether Sfp1 could regulate TORC1 activity toward Sch9. To this end, we used a chemical fragmentation assay that measures the in vivo phosphorylation of TORC1-dependent sites in the Sch9 C terminus, which is strongly diminished by rapamycin treatment, but increased after the addition of CHX (Urban et al., 2007) (Figure 6A). Notably, we observed a strong increase in Sfp1 phosphorylation in sfp1D cells (Figure 6A). Rapamycin still diminished Sch9 phosphorylation in sfp1D cells, and CHX further increased the Sch9 phosphorylation in these cells. Overexpression of SFP1 from the GAL1 promoter strongly reduced the phosphorylation of Sch9 (Figures 6B and S6A), causing a simultaneous increase in cell size (Figure S6B). Together, these results indicate that Sfp1 protein levels strongly affect the activity of TORC1 toward Sch9 and suggest the existence of a feedback mechanism linking the activity of Sfp1 and Sch9 through TORC1. Consistent with this proposed feedback mechanism, we also detected an increase in Sch9 phosphorylation in sfp1-1 cells (Figure S6C).

We considered two models to explain this feedback regulation: Sfp1 regulates TORC1 activity either via a direct interaction,
or through Sfp1 downstream functions (i.e., RP/Ribi gene activation) (Fingerman et al., 2003; Jorgensen et al., 2004; Marion et al., 2004). To discriminate between the two models, we have employed SFP1 mutants that contain mutations in the region of the two Zn fingers in the Sfp1 C terminus (Fingerman et al., 2003) that abolish the ability of the protein to activate the transcription of an Sfp1-dependent reporter gene (Fingerman et al., 2003). Sch9 was hyperphosphorylated in strains containing these Zn-finger mutations (Figure 6C). Furthermore, the mutants showed reduced binding to an RP gene promoter (Figure 6D), but did not affect the interaction between Sfp1 and TORC1 (Figure 6E). Taken together, these results indicate that Sfp1 does not regulate TORC1 activity through a direct interaction, but instead suggest that feedback is transmitted through the transcriptional regulatory function of Sfp1, i.e., the activation of RP/Ribi gene transcription. This model is supported by the observation that SCH9 mutations also cause hyperactivation of TORC1 toward the Sch9 C terminus (Figure 6F). When the TORC1-dependent phosphorylation sites of Sch9 were mutated to alanine (sch9-5A; Urban et al., 2007) we detected
Figure 6. Sfp1 Regulates TORC1 Activity toward Sch9

(A) Deletion of SFP1 hyperactivates TORC1. Sch9 phosphorylation in wild-type and sfp1Δ cells containing Sch9-5HA plasmid was measured by using NTCB chemical fragmentation analysis (Urban et al., 2007). Sch9 is dephosphorylated in response to rapamycin (rapa; 200 ng/ml, 10 min) and hyperactivated by CHX (25 μg/ml, 10 min). Sch9-5HA was detected by anti-HA western. Relative ratios (normalized to untreated wild-type sample) between phosphorylated (+P) and dephosphorylated (−P) fractions of Sch9 are shown below the lanes.

(B) Sfp1 overexpression represses TORC1 activity. Sfp1 was overexpressed by adding galactose (2%) for indicated times to cells containing an integrated GAL1-promoter-driven SFP1 (GAL1-SFP1) and a plasmid expressing Sch9-5HA. An otherwise isogenic strain (SFP1Δ) lacking the GAL1-SFP1 construct was used as control. The kinetics of Sfp1 expression from the GAL1 promoter is shown in Figure S6A.

(C) Mutations in the C-terminal Zn-finger region of Sfp1 hyperactivate TORC1-dependent phosphorylation of Sch9. Sch9 phosphorylation in wild-type and indicated SFP1 mutant cells containing a Sch9-5HA-expressing plasmid was measured by using NTCB chemical fragmentation analysis (as before).

(D) Reduced binding of Sfp1 Zn-finger mutants to an RP gene promoter. ChIP analysis of SFP1 indicated whether the Sfp1-Mrs6 interaction regulates Sfp1 function, we compared three alleles and Kog1-3HA were subjected to single-step TAP purification and analyzed as in Figure 2A.

(E) Sfp1 Zn-finger mutations do not affect the TORC1 interaction. Lysates from cells expressing the indicated TAP-tagged SFP1 alleles and Kog1-3HA were subjected to single-step TAP purification and analyzed as in Figure 2A.

(F) Effect of Sch9 mutations on TORC1 activity toward the Sch9 C terminus. TORC1 activity toward an sch9-T570A-6HA reporter construct was assayed by using NTCB chemical fragmentation analysis (as before). In addition to the catalytically inactive Sch9 reporter, cells expressed an additional allele of SCH9 as indicated.

Molecular Cell
TORC1 Signaling to Sfp1 in Ribosome Biogenesis

hyperphosphorylation of a 6HA-tagged sch9 protein. (The catalytically inactive sch9-T570A mutant protein was used as the "reporter" for this experiment, since it is completely inactive in downstream signaling, but still contains fully functional TORC1 target sites [Urban et al., 2007]). We also found that rapamycin-resistant SCH9 mutants (sch9-3E and sch9-2D3E) exhibit a mild hyperactivation of TORC1-mediated Sch9 phosphorylation (Figure 6F), suggesting that they too are not fully functional in downstream signaling.

Mrs6 Regulates Sfp1 and Sch9 Phosphorylation

Mrs6, an essential Rab escort protein involved in intracellular vesicular trafficking (Benito-Moreno et al., 1994; Fujimura et al., 1994), was identified as a prominent Sfp1-interacting protein, appearing to bind to Sfp1 in ~1:1 stoichiometry (Figure 1). The Sfp1-Mrs6 interaction has also been identified in two different large-scale protein interaction screens (Ho et al., 2002; Tarassov et al., 2008). We confirmed this in vivo interaction by showing that a HA-tagged version of Mrs6 was specifically coimmunoprecipitated with Sfp1-TAP (Figure S7A).

Unlike the case of TORC1, this interaction was not affected by rapamycin treatment (Figure S7A). By comparing the input levels to IP material, we estimate that 50%–100% of Sfp1 is bound by Mrs6 in cells, but since Mrs6 is highly abundant in cells, only 5%–10% of the cellular Mrs6 pool is bound by Sfp1.

To test if Mrs6 affects Sfp1 function, we used a temperature-sensitive (ts) lethal MRS6 mutant (mrs6-2), which shows functional defects already at the permissive temperature (25°C) (Bialek-Wyrzykowska et al., 2000). Sfp1 was strongly dephosphorylated, and its interaction with TORC1 was reduced in mrs6-2 cells at 25°C; a shift to the nonpermissive temperature showed no further effects (Figures 7A and S7B). Sfp1 nuclear concentration was also reduced in mrs6-2 cells compared to wild-type at the permissive temperature, and shifting cells to 37°C led to a further increase in cytoplasmic Sfp1 levels (Figure 7B).

Mrs6 could regulate Sfp1 either through the interaction between the two proteins or by regulating TORC1. To test whether the Sfp1-Mrs6 interaction regulates Sfp1 function, we compared three SFP1 mutants, sfp1-Zn2-3A, sfp1-Zn3-4A, and sfp1-Δ627-656 (Fingerman et al., 2003). Although all three mutations cause a severe defect in an assay for the transcriptional activation function of Sfp1 (Fingerman et al., 2003), we found that the Zn2-3A and Zn3-4A mutant proteins showed a severe interaction defect with Mrs6, whereas the Δ627–656 mutant binds to Mrs6 (Figure 7C). Notably, GFP-tagged
versions of sfp1-Zn2-3A and sfp1-Zn3-4A displayed a strong reduction in nuclear localization, whereas sfp1-Δ627–656 was indistinguishable from wild-type in this assay (Figure 7D). Nevertheless, all three mutations caused increased TORC1-dependent phosphorylation of Sch9 (Figures 6C and 8C). Taken together, these results suggest that the Sfp1-Mrs6 interaction is important for the correct nuclear localization of Sfp1 and lend further support to the notion that the transcriptional activation function of Sfp1 feeds back on TORC1-dependent phosphorylation of Sch9.

To test if Mrs6 regulates TORC1 activity, we looked at TORC1-dependent phosphorylation of Sch9 in mrs6-2 mutant cells (Figure 7E). Phosphorylation of the Sch9 C terminus was reduced in mutant cells at the permissive temperature and was essentially undetectable after 2 hr at 37 °C. This result suggests that Mrs6 might regulate TORC1 activity (at least toward Sch9) in a manner that is independent of its interaction with Sfp1.

**DISCUSSION**

Here, we provide evidence that Sfp1, a transcriptional activator of RP and Ribi genes, is associated with Tor1 kinase and other TORC1 proteins. Furthermore, we show that TORC1 phosphorylates Sfp1 on multiple residues, both in vitro and in vivo, and that mutation of these residues leads to Sfp1 localization and RP promoter-binding defects. Phosphorylation of Sfp1 by TORC1 is strongly inhibited by rapamycin, caffeine, and wortmannin, and the first two agents also significantly reduce the Sfp1-TORC1 physical interaction. Interestingly, neither nutrient depletion nor osmotic stress reduces Sfp1-TORC1 association or TORC1 phosphorylation of Sfp1, whereas both of these conditions cause rapid dephosphorylation of Sch9 kinase, another TORC1 substrate (Urban et al., 2007). Furthermore, we uncovered an inverse relationship between TORC1 phosphorylation of Sch9 and Sfp1 levels, suggesting the existence of a homeostatic mechanism regulating the activity of TORC1 toward two key substrates that control ribosome biogenesis, Sch9 and Sfp1. Finally, we provide evidence that the Sfp1-interacting protein Mrs6, an essential Rab escort protein, is required for proper nuclear localization of Sfp1 and, in addition, regulates TORC1 activity through a different mechanism. A model summarizing the results is presented in Figure 7E.

**Regulation of Sfp1-TORC1 Association and Subcellular Localization**

In optimally growing cells, Sfp1 appears predominantly nuclear and localizes (as measured by ChIP) to a number of RP gene promoters (Jorgensen et al., 2004; Marion et al., 2004). By contrast, unfavorable nutrient conditions or stress lead to a rapid relocation of Sfp1 from the nucleus to the cytoplasm. Our finding that under optimal growth conditions a significant amount of Sfp1 is associated with TORC1 suggests that a fraction of cellular TORC1 may act directly in the nucleus together with Sfp1 to promote RP and Ribi gene transcription in rapidly growing cells. This model is supported by a recent study that finds Tor1 concentrated in the nucleus in rapidly growing cells (Li et al., 2006) and, by our observation, that increased TOR association, after CHX treatment, is correlated with increased Sfp1 nuclear localization. An alternative explanation of our data, which we cannot presently rule out, is that the Sfp1-TORC1 interaction in growing cells corresponds to a small cytoplasmic or membrane-bound fraction of Sfp1, and that phosphorylation of Sfp1 in the cytoplasm by TORC1 directs Sfp1 to the nucleus.

We found that TORC1 association with and phosphorylation of Sfp1 were unaffected by either nutrient deprivation or osmotic stress, conditions in which phosphorylation of another TORC1 substrate, Sch9, is strongly and rapidly reduced (Urban et al., 2007). Instead, as reported previously (Jorgensen et al., 2004; Marion et al., 2004), we found that the bulk of Sfp1 exited the nucleus under these conditions and accumulated in the cytoplasm. Given the fact that the TORC1-Sfp1 association remained unchanged during this translocation of Sfp1, we propose that the TORC1-Sfp1 complex itself is exported from the nucleus, although direct proof of this is still lacking. We note, however, that Li et al. (2006) demonstrated that the bulk of nuclear Tor1 does indeed leave the nucleus and accumulate in the cytoplasm upon nutrient withdrawal. In summary, our data suggest that nutrient removal and stress regulate TORC1 signaling through different mechanisms than rapamycin or other direct small-molecule inhibitors.

Although treatment with three different TORC1 small-molecule inhibitors (rapamycin, caffeine, and wortmannin) leads to rapid Sfp1 dephosphorylation and nuclear exit, our results suggest that these inhibitors have different modes of action with respect to phosphorylated substrate. Rapamycin strongly inhibits the TORC1-Sfp1 interaction. Caffeine also inhibits the interaction, but to a lesser extent compared with rapamycin. Wortmannin has little or no effect on the interaction, which is consistent with the known action of wortmannin (inhibition by covalent binding to the ATP-binding site [Jazayeri et al., 1999]), which would not be expected to interfere with substrate recognition. It is interesting to note that rapamycin also abrogates the TORC1-Tap42 interaction, as well as TORC1 phosphorylation of Tap42 (Yan et al., 2006), analogous to our observations with Sfp1. Taken together, these results suggest that in vivo rapamycin regulates TORC1 at the level of substrate recognition/binding, at least in part.

**Sfp1 Regulation of TORC1 and Sch9**

Both Sch9 and Sfp1 act as positive regulators of RP and Ribi gene expression (Fingerman et al., 2003; Jorgensen et al., 2004; Marion et al., 2004; Urban et al., 2007), apparently working through parallel pathways (Jorgensen et al., 2004; Figures 5C, 5D, 5S, and 5S). Our finding that increased Sfp1 expression leads to reduced phosphorylation of Sch9 by TORC1, and that sfp1Δ cells display Sch9 hyperphosphorylation, points to the existence of a negative-feedback mechanism connecting the Sfp1 and Sch9 pathways (Figure 7F). The importance of this feedback regulation is highlighted by the observation that mutation of TORC1 phosphorylation sites in Sch9 (sch9-5A) (Urban et al., 2007) is lethal in combination with SFP1 deletion (Figure S6D), indicating that TORC1 phosphorylation of Sch9 is essential for sfp1Δ cell survival. Sch9 is also hyperphosphorylated in sfp1-1 cells (Figure S6C), although to lesser extent than in sfp1Δ. This is consistent with the milder phenotypes of
**Figure 7. Mrs6 Regulates TORC1 Signaling to Sfp1 and Sch9**

(A) Reduced phosphorylation and TORC1 binding of Sfp1 in mrs6-2 cells. Sfp1-TAP was purified from MRS6 or mrs6-2 cells at indicated temperatures. Cells were treated with rapamycin (rapa; 200 ng/ml, 45 min) or drug vehicle prior to harvesting. Samples were analyzed as in Figure 3A. Protein inputs for the experiment are shown in Figure S7 B.

(B) Reduced nuclear localization of Sfp1 in mrs6-2 cells. Cells carrying a genomic GFP-tagged SFP1 and mCherry-tagged HHF2 in MRS6 or mrs6-2 backgrounds were used for the experiment at indicated temperatures. Details of microscopy and quantification are described in Figure 3C. Quantification data are reported as averages (bars) from two experiments, with standard deviations indicated by the lines above.

(C) Interaction of sfp1 Zn-finger mutants with Mrs6. Lysates from cells expressing the indicated TAP-tagged SFP1 alleles and Mrs6-3HA were subjected to single-step TAP purification and analyzed as described in Figure 2 A.
sfp-1 mutation compared to SFP1 deletion, and it indicates that sfp-1 protein is still partially functional even though most (if not all) TORC1 target sites have been mutated. Sfp1-1 may, for example, be activated by other signaling inputs (e.g., the PKA pathway). Furthermore, TORC1 still interacts with sfp1-1 in a wild-type manner (Figure S5A) and may regulate Sfp1 function through a second, phosphorylation-independent mechanism.

To address the mechanism of Sfp1 feedback regulation on TORC1-dependent Sch9 phosphorylation, we examined two SFP1 mutants with alterations in or nearby a conserved C-terminal Zn-finger domain, both of which are defective in Sfp1-dependent transcription and RP gene promoter binding (Fingerman et al., 2003) (Figure 6D). Our finding that these mutant proteins bind to TORC1 in a wild-type manner (Figure 6E) yet display sfp1J-like Sch9 hyperphosphorylation (Figure 6C), suggests that the Sfp1-TORC1 interaction per se is not sufficient for feedback, but rather that Sfp1 promoter binding and transcriptional activation are required. Since Sfp1 is known to regulate ribosome assembly and translation efficiency (Fingerman et al., 2003), presumably via transcriptional activation of RP and Ribi genes, one possible explanation for the feedback mechanism would implicate the downstream effects of SFP1 on ribosome biogenesis, which might be sensed by TORC1 through the same (currently uncharacterized) pathway that responds to translation inhibition by CHX (Figure 7F). Consistent with this model, we see that in SFP1-overexpressing cells, where Sch9 phosphorylation is diminished, treatment with CHX results in rapid Sch9 hyperphosphorylation, to a similar level as in CHX-treated wild-type cells (Figure S6E).

Further evidence that RP/Ribi gene expression feeds back on TORC1-dependent Sch9 phosphorylation comes from our finding that a loss-of-function mutation in SCH9 itself (sch9-5A) (Urban et al., 2007) also leads to hyperactivation of TORC1 activity toward Sch9 (Figure 6F). Since activation of RP/Ribi gene transcription is the only known common output of Sfp1 and Sch9 function, it seems likely that this is the link from Sfp1 and Sch9 to regulation of TORC1 activity. Since different Sch9 mutant proteins do not affect Sfp1 phosphorylation or localization (Figure S3), this feedback seems to be specific to TORC1 phosphorylation of Sch9 and does not regulate TORC1 signaling to Sfp1, again suggesting functional differences in TORC1 signaling to Sfp1 and Sch9 (Figure 7F).

**Mrs6 and TORC1 Signaling**

Mrs6 is an essential protein, which has been characterized as a Rab escort protein and has been shown to be involved in intracellular vesicular trafficking (Benito-Moreno et al., 1994; Fujimura et al., 1994). Here, we present evidence suggesting that a large fraction (perhaps all) of the cellular pool of Sfp1 binds Mrs6, and that Mrs6 promotes both Sfp1 phosphorylation and nuclear localization (Figure 7). These data predict that Mrs6 might also promote the negative feedback of Sfp1 on TORC1-dependent Sch9 phosphorylation. However, we found instead that Sch9 phosphorylation is strongly reduced in the mrs6-2 mutant strain, suggesting that Mrs6 acts as a positive regulator of TORC1, presumably through an Sfp1-independent mechanism. Mrs6 thus appears to influence at least two different nodes of TORC1 signaling (Figure 7F).

Membrane trafficking has been linked to TORC1 signaling previously through genetic interactions between TORC1 and membrane trafficking components (Aronova et al., 2007; Zurita-Martinez et al., 2007). In addition, Sfp1 nuclear localization has been linked to membrane sorting by the observation that the secretory pathway inhibitor tunicamycin, which causes RP gene transcriptional downregulation through a PKC-dependent pathway (Nierras and Warner, 1999), leads to a gradual decline in Sfp1 nuclear localization (Jorgensen et al., 2004). We have also observed that tunicamycin treatment provokes a decrease in Sfp1 phosphorylation (Figure S8A), suggesting that tunicamycin/PKC-dependent inhibition of RP gene transcription may function via TORC1. Consistent with this model, tunicamycin also inhibits the TORC1-dependent phosphorylation of Sch9 (Figure S8B). In summary, our findings thus considerably strengthen and expand connections between membrane trafficking and TORC1 signaling.

Although TORC1 (Wullschleger et al., 2006), Sch9 (a mammalian S6K1 ortholog [Urban et al., 2007]), and Mrs6 (Benito-Moreno et al., 1994; Fujimura et al., 1994) are highly conserved, no obvious Sfp1 ortholog has been identified in multicellular organisms. However, the proto-oncogene c-Myc, one of the few known mammalian regulators of RP and Ribi gene transcription (reviewed in Dang et al., 2006), shares several properties with Sfp1. For example, c-Myc function is linked to mTOR and PKA signaling (reviewed in de Nigris et al., 2006), and c-Myc overexpression leads to increased cell size and increased expression of genes encoding ribosomal and nucleolar proteins (Kim et al., 2000), effects remarkably similar to that of Sfp1 overexpression (Jorgensen et al., 2004) (Figure S6). Taken together with data reported here, these analogies provide further support to the idea that Sfp1 is the budding yeast functional analog of mammalian c-Myc (Cook and Tyers, 2007). A further understanding of Sfp1 function may thus have broad implications for growth regulation of ribosome biogenesis in multicellular organisms.

**Experimental Procedures**

**Strains and Plasmids**

Yeast strains and plasmids used in this study are listed in Tables S1 and S2.

**Single-Step TAP Tag Purifications**

Yeast pellets were resuspended in an equal volume of lysis buffer (100 mM HEPES-KOH [pH 8.0], 10% glycerol, 10 mM EGTA, 0.1 mM EDTA, 0.4%
NP-40, 60 mM NaOAc, 1 mM PMSF, 1 mM DTT, and 1x protease inhibitor cocktail [Roche] and were lysed by vortexing in the presence of an equal volume of zirconia/silica beads by using a Beadbeater (Biospec Products) at +4°C. Cell lysates were cleared with a 10 min, 3000 rpm spin at +4°C. For immunoprecipitation, Rabbit IgG (Sigma I5006)-coupled magnetic epoxy beads (Dynabeads M-270; Dynal Biotech ASA, Invitrogen) were added, and tubes were rotated for 3 hr at 4°C. Beads were collected with a magnet and washed three times with lysis buffer. Proteins were eluted with SDS sample buffer and separated by SDS-PAGE. Further details including details of mass spectrometry, immunoprecipitations, and in-gel phosphorylation measurements are available below and in the Supplemental Data.

Fluorescence Microscopy

GFP and mCherry were visualized directly in living cells of strains with integrated SFP1-GFP and HHF2-mChERRY without fixation. Cells were grown in riboflavin-free SC medium, and stress conditions were applied as specified for individual experiments in the figure legends. Images were recorded on a Leica AF6000 LX widefield microscope with a CoolSnap HQ camera (Photometrics, Roper Scientific, CA). The quantification of colocalization was done with Imaris software (Bitplane AG, Switzerland, Zurich).

Coimmunoprecipitation Assays

Overnight cultures were diluted in 250 ml fresh YPAD to an OD 600 of 0.2 and were grown to an OD 600 of 1.0. Lysates were prepared as described above, then diluted with lysis buffer to 5 mg/ml protein (10 mg total in 2 ml). Input samples were drawn at this point by mixing 50 μl lysate with an equal volume of 2x SDS loading buffer. For immunoprecipitations, 40 μl Rabbit IgG (Sigma I5006)-coupled magnetic epoxy beads (Dynabeads M-270) were added, and tubes were rotated for 3 hr at 4°C. Beads were collected with a magnet and washed three times with 1 ml lysis buffer. Proteins were eluted with 1x SDS-PAGE sample buffer (10 min at 65°C), separated by SDS-PAGE, and then electroblotted onto nitrocellulose membranes. The membranes were blocked in PBS containing 5% milk and 0.1% Tween 20, then incubated with the primary antibody 12CA5 for HA epitope detection, (diluted 1:5000) and anti-TAP (Open Biosystems) for TAP-Tag detection (diluted 1:10000) in blocking solution (PBS plus 2.5% milk). The membranes were subsequently washed, and tagged proteins were detected with horseradish peroxidase-conjugated secondary antibodies (anti-mouse for 12CA5 and anti-rabbit for anti-TAP) and ECL reagents (Amersham Pharmacia Biotech). Anti-Act1 (Abcam ab8224) antibody was used to detect Act1 protein.

In-Gel Measurement of Sfp1 Phosphorylation

For experiments in which Sfp1 phosphorylation and Tor1 coimmunoprecipitation were detected simultaneously, 400 ml YPAD cultures were used. To avoid loss of phosphorylation during sample preparation, cells were collected by centrifuging gently (RT, 4000 rpm, 5 min) and frozen immediately after pouring off the supernatant by addition of liquid nitrogen. Lysis buffer was accompanied by 0.1x PPI mix. For immunoprecipitations, samples were diluted with lysis buffer to 5 mg/ml (20 mg protein was adjusted to 4 ml with lysis buffer plus inhibitors). For λ-phosphatase treatment, beads were washed one more time with wash buffer (1x PBS, 10% [v/v] glycerol, 0.5% [v/v] Tween 20) after which beads were incubated with λ-PPase (New England Biolabs) for 25 min at 30°C; for λ-PPase + phosphatase inhibitor treatment, reaction mixture was accompanied by 1x PPI. Electrophoresis and western blot detection were performed as described above. The quantitative Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) and SYPRO Ruby protein stain (Bio-Rad) in-gel stains were used in accordance with the manufacturers’ protocols. The fluorescent intensity of protein bands was visualized and quantified by using an Ettan DIGE Imager and ImageQuant TL software (GE Healthcare). Sfp1 phosphoprotein levels were normalized to total Sfp1 protein levels and to the background in each lane and are presented as relative phosphorylation normalized to untreated wild-type sample.

Kinase Assays

Sfp1 was purified from TAP-tagged strains (HL38/HL226/HL227) grown to an OD 600 of ~3.5 (250 ml preassay point). Purification was done as for the coimmunoprecipitations experiment. Beads were washed three times with lysis buffer and once with wash buffer (1x PBS, 10% [w/v] glycerol, 0.5% [v/v] Tween 20). Sch9-3HA used in a kinase assay (Figure S3C) was purified as described previously (Urban et al., 2007) and added to the kinase reaction. Kinase reactions were performed and analyzed as described (Urban et al., 2007). Briefly, kinase reactions were performed in kinase buffer (1x PBS, 15% glycerol, 0.5% Tween 20, 4 mM MgCl2, 10 mM DTT, and 2 μg/ml heparin). Drugs or drug vehicle were added just prior to starting the reaction. FKBP12 protein was produced in Escherichia coli from a pGEX-6P-FPR1 vector (pAUS) and was added with rapamycin to a final concentration of 20 ng/μl. Assays were started with the addition of 100 μM ATP and 50 μC [γ-32P]ATP, shaken for 20 min at 30°C, and terminated with the addition of 8 μl 5x SDS-PAGE sample buffer. Samples were heated at 95°C for 5 min before proteins were resolved on an SDS-PAGE gel, stained with Coomassie to detect total protein levels, and phosphorylation was detected by autoradiography, which was analyzed by using a BioRad Molecular Imager.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Blanchi et al., 2004), with the following exceptions. For immunoprecipitations, 20 μl Rabbit IgG (Sigma I5006)-coupled magnetic epoxy beads (Dynabeads M-270; Dynal Biotech ASA, Invitrogen) were added, and tubes were rotated for 3 hr at 4°C. Immunoprecipitates were washed with lysis buffer. Both an aliquot of sonicated cleared extract (input) and the immunoprecipitated material were de-crosslinked in TE plus 1% SDS for at least 8 hr at 65°C. Quantification of immunoprecipitated DNA was obtained by real-time PCR by using SYBR Green detection on an Applied Biosystems ABI Prism 7700 machine. Primers used were RPL2B-5′ (CACAGAGGTGCTGCCAGCT) and RPL2B-3′ (GCAGAATCCACCAAGGATGT) for the RPL2B promoter and H2L228 (GAATTGAGGTGCGGGCTAAGA) and H2L229 (AGAACCGTCGAAACCCTT GAAA) for the ACT1 gene. For each data point, results were obtained from at least three measurements.

Cell Size Measurement

Approximately 0.4 x 10^6 cells from log-phase cultures were diluted in 10 ml Casadyn (Schräfe System GmbH, Reutlingen, Germany), sonicated gently for 15 s to dissociate cell aggregates, and analyzed with a Casy Model TT Cell Counter + Analyzer System (Schräfe System GmbH, Reutlingen, Germany). To eliminate background, particles < 2.50 μm or > 18.55 μm diameter were excluded from the analysis.

Rapamycin and Cycloheximide Sensitivity “Hallo” Assays

Overnight cultures (100 ml) were streaked on YPAD plates, and 5 μl CHX (10 mg/ml) or 10 μl rapamycin (1 mg/ml) was pipetted onto the middle of the plate. Plates were photographed after 4 days of growth at 30°C. The area of the inhibition (cm²) was measured for three independent cultures and is shown as average values (bars) with standard deviations indicated by the lines above. A two-tailed, paired Student’s t test was performed, and p values were calculated comparing the indicated strains (‘p < 0.05; **p < 0.01; ***p < 0.001).

Other Experimental Procedures

Details of strains, mass spectrometry, and NTGB chemical/fragmentation analysis of Sch9 are described in the Supplemental Data.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, eight figures, and four tables and are available at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00099-9.

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