Antagonistic interactions between the cAMP-dependent protein kinase and Tor signaling pathways modulate cell growth in Saccharomyces cerevisiae

Vidhya Ramachandran\textsuperscript{1,2} and Paul K. Herman\textsuperscript{1,2,3}

\textsuperscript{1} Department of Molecular Genetics
\textsuperscript{2} Program in Molecular, Cellular and Developmental Biology
\textsuperscript{3} To whom correspondence should be addressed.

The Ohio State University, Columbus Ohio 43210
Running title: Interactions between the Tor and PKA pathways

Keywords: TORC1 signaling/ PKA/ Ras proteins/ Cki1 choline kinase/ Genetic buffering

Corresponding author:

Paul K. Herman, Ph.D. Phone: (614) 688-5581
Department of Molecular Genetics Fax: (614) 292-4466
The Ohio State University E-mail: herman.81@osu.edu
484 West Twelfth Avenue, Room 984
Columbus, Ohio 43210.
ABSTRACT

Eukaryotic cells integrate information from multiple sources in order to respond appropriately to changes in the environment. Here, we examined the relationship between two signaling pathways in *Saccharomyces cerevisiae* that are essential for the coordination of cell growth with nutrient availability. These pathways involve the cAMP-dependent protein kinase (PKA) and Tor proteins, respectively. Although these pathways control a similar set of processes important for growth, it was not clear how their activities were integrated *in vivo*. The experiments here examined this coordination and, in particular, tested whether the PKA pathway was primarily a downstream effector of the TORC1 signaling complex. Using a number of reporters for the PKA pathway, we found that the inhibition of TORC1 did not result in diminished PKA signaling activity. To the contrary, decreased TORC1 signaling was generally associated with elevated levels of PKA activity. Similarly, TORC1 activity appeared to increase in response to lower levels of PKA signaling. Consistent with these observations, we found that diminished PKA signaling partially suppressed the growth defects associated with decreased TORC1 activity. In all, these data suggested that the PKA and TORC1 pathways were functioning in parallel to promote cell growth but that each pathway might restrain, either directly or indirectly, the activity of the other. The potential significance of this antagonism for the regulation of cell growth and overall fitness is discussed.
Eukaryotic cells respond to a variety of signals, including growth factors and essential nutrients, by activating specific signal transduction pathways. Although these pathways are often studied in isolation, most cells are exposed to a number of different signals at any one time. Cells must therefore be able to appropriately integrate the information coming from these multiple sources. This calculus is further complicated by the fact that these signaling pathways may interact with each other to modulate the intracellular response. A complete understanding of eukaryotic biology therefore will require a thorough knowledge of these latter interactions and how they influence signal transduction.

Cell growth in the budding yeast, *Saccharomyces cerevisiae*, is controlled primarily by nutritional cues. These inputs are interpreted by a variety of signaling pathways that allow for the appropriate growth response to the conditions present. Two of the best-characterized of these pathways involve the Tor proteins and the cAMP-dependent protein kinase (PKA) (BAHN et al. 2007; DECHANT and PETER 2008; ZAMAN et al. 2008). The inactivation of either of these pathways results in an arrest within a G0-like resting state, known as stationary phase (GRAY et al. 2004; HERMAN 2002; IIDA and YAHARA 1984; SCHNEPER et al. 2004). In addition, mutants with constitutive, or unregulated, levels of PKA activity are unable to arrest normally in this resting state upon nutrient deprivation (BROACH 1991; BROEK et al. 1985). Cells in stationary phase exhibit a diminished rate of metabolism and elevated levels of particular catabolic processes, such as autophagy (GRAY et al. 2004; HERMAN 2002; WERNER-WASHBURNE et al. 1993). These, and related observations, have led to the suggestion that these two pathways control the transitions between active division and quiescence (HERMAN 2002; ZAMAN et al. 2008).
In *S. cerevisiae*, the intracellular level of cAMP is controlled via two routes involving either the small GTP-binding Ras proteins, Ras1 and Ras2, or the Gα protein, Gpa2 (KUBLER et al. 1997; TODA et al. 1985; ZAMAN et al. 2008). The former path involving the Ras proteins seems to be the most important for the PKA effects on cell growth (SANTANGELO 2006; WANG et al. 2004). The active, GTP-bound form of these Ras proteins directly interact with the adenylyl cyclase, Cyr1, and stimulate the production of cAMP (FIELD et al. 1990; SUZUKI et al. 1990). This cAMP is bound by the regulatory subunit of PKA, Bcy1, causing this inhibitory protein to dissociate from the catalytic subunits (TODA et al. 1987a; UNO et al. 1982). These catalytic subunits are then free to phosphorylate their respective targets and thereby exert their influence on cell physiology (BROACH 1991; HERMAN 2002; TODA et al. 1987b). The Tor proteins are themselves serine/threonine-specific protein kinases that play a role in coordinating growth with specific environmental cues in all eukaryotes (DE VIRGILIO and LOEWITH 2006a; GUERTIN and SABATINI 2007; WULLSCHLEGER et al. 2006). The Tor proteins exist in two complexes, known as TORC1 and TORC2, that are thought to control distinct processes important for cell growth (LOEWITH et al. 2002; WEDAMAN et al. 2003). TORC1 is sensitive to the macrolide, rapamycin, and influences protein synthesis, general metabolism and transcription by RNA polymerases I and III (GINGRAS et al. 2004; HEITMAN et al. 1991; PROUD 2007; WULLSCHLEGER et al. 2006). In contrast, TORC2 has been implicated in functions concerned with the organization of the actin cytoskeleton (CYBULSKI and HALL 2009; JACINTO et al. 2004). However, recent work has indicated that the functional differences between these complexes may not be so clear-cut (ROHDE et al. 2008).

Previous studies have indicated that the PKA and TORC1 pathways control a similar set of biological processes in *S. cerevisiae*. In particular, both pathways positively regulate
processes necessary for growth, such as protein translation, and inhibit others that are associated
with growth-arrested cells, like autophagy and specific stress responses (DeChant and Peter
2008; Zaman et al. 2008). However, the underlying reasons for this functional overlap remain
unclear. In particular, there are conflicting reports concerning the order of action of these two
pathways. For example, several studies have suggested that these pathways function
independently to control a similar set of targets (Lipman and Broach 2009; Pedruzi et al.
2003; Stephan et al. 2009; Zurita-Martinez and Cardenas 2005). In contrast, other
investigators have suggested that the PKA pathway is a downstream effector of TORC1
signaling activity (Schmelzle et al. 2004; Soulard et al. 2010). In addition, the identities of
the upstream regulators for each pathway have not yet been unequivocally determined, although
the current data do suggest that each responds to different nutritional cues (Bahn et al. 2007;
DeChant and Peter 2008; Zaman et al. 2008). The Ras/PKA pathway, for example, is thought
to respond to the available levels of fermentable sugars, and perhaps to intracellular glucose, in
particular (Mbonyi et al. 1988; Santangelo 2006; Slattery et al. 2008; Wang et al. 2004;
Zaman et al. 2009). In contrast, the Tor pathway appears to respond to nitrogen availability, and
perhaps specifically to particular amino acids (De Virgilio and Loewith 2006a; Jacinto and
Lorberg 2008; Kim et al. 2008; SancaK et al. 2008; Wullschleger et al. 2006). Determining
how these two pathways interact is essential for a complete understanding of the regulation of
growth in this budding yeast.

The primary goal of this study was to define the relationship between the Tor and
Ras/PKA pathways in S. cerevisiae. In particular, we set out to test whether the Ras/PKA
pathway was a downstream effector of TORC1 signaling activity. The reporters used for these
studies assessed either the phosphorylation status of particular PKA substrates or the activation
state of the Ras proteins. In all, the data here are most consistent with these two signaling pathways functioning in parallel to control cell growth. However, although neither pathway was found to be down-regulated by the inactivation of the other, we did find evidence of potential interactions occurring between these pathways. Interestingly, these interactions appeared to be mutually antagonistic in nature. For example, we found that the level of PKA phosphorylation on multiple substrates was elevated following rapamycin treatment. Moreover, the TORC1-dependent phosphorylation on Atg13 increased in response to diminished Ras/PKA signaling. Finally, we detected genetic interactions between PKA and Tor mutations that were consistent with the antagonistic relationship suggested by the molecular readouts of pathway activities. Models describing how these interactions between the PKA and Tor pathways might occur and how they might influence the robustness of yeast growth are discussed.

MATERIALS AND METHODS

Yeast strain construction and growth conditions: The yeast strains used in this study are listed in Table 1. The strains used for most of the protein analyses were PHY1220, PHY1682 and Y3175. Standard *E. coli* growth conditions and media were used throughout this study. The yeast rich growth medium, YPAD, consists of 1% yeast extract, 2% Bacto-peptone, 500 mg/L adenine-HCl, and 2% glucose. The yeast YM glucose and SC glucose minimal growth media have been described (CHANG et al. 2001; KAISER et al. 1994). The nitrogen starvation medium, SD-N, consists of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate, and 2% glucose. Growth media reagents were from DIFCO. Strains carrying the *MET3-RAS2*<sup>val19</sup> or *MET3-RAS2*<sup>ala22</sup> alleles were grown in medium containing 500 µM methionine to keep the *MET3* promoter in its repressed state. Expression from the *MET3* promoter was induced by
transferring cells to a medium that lacked methionine. Expression from the \textit{CUP1} promoter was induced by the addition of 100 \(\mu\)M CuSO\(_4\) to the growth medium.

**Plasmid construction:** The plasmid, pPHY921, consists of the \textit{RAS2}\textsuperscript{val19} allele cloned into pRS416. The \textit{LEU2}-marked \textit{MET3-RAS2}\textsuperscript{val19} plasmid, pPHY795, was constructed as described (Howard \textit{et al.} 2002). Within the context of this construct, site-directed mutagenesis was used to introduce the G22A alteration for this study. The \textit{URA3}-marked high-copy \textit{PDE2} plasmid, pM387 (or pPHY1107) was generously provided by Dr. M. Hampsey. The plasmid vectors used for the copper-inducible expression of the epitope-tagged versions of Tpk1 and the substrate proteins have been described (Deminfof \textit{et al.} 2006). Tpk1 was tagged at its N-terminus with three copies of the HA epitope. The pGEX-RBD plasmid encodes amino acids 1 - 149 of Raf-1 fused in frame to GST in the vector pGEX-2T (Taylor and Shalloway 1996). The Cki1 reporter construct, pPHY2328, consisted of the N-terminal 200 amino acids of the Cki1 substrate protein, tagged with six copies of the Myc epitope at its N-terminus (Deminfof \textit{et al.} 2006). The Cki1 variants described herein were constructed by site-directed mutageneses of pPHY2328 performed with the Transformer mutagenesis kit (Clontech). A Protein A-tagged Cki1 construct under the control of the promoter from the \textit{ADH1} gene was made by subcloning the Cki1 open reading frame from pPHY2328 into the previously-described plasmid, pPHY1044 (Budovskaya \textit{et al.} 2002). The Rim15 reporter, pPHY2272, encoded a fragment consisting of residues 1431 – 1671 that were tagged with six copies of the myc epitope at its N-terminus (Deminfof \textit{et al.} 2006). The plasmid, pPHY2426, encoded an HA epitope-tagged version of Atg13 under the control of the promoter from the copper-inducible \textit{CUP1} gene, and was described previously (Stephan \textit{et al.} 2009). The \textit{SRB9} plasmid, pPHY1066, was generously
provided by Dr. Marian Carlson and was originally named pWS121. This plasmid encodes an HA epitope-tagged, full-length Srb9 that is under the control of the promoter from the yeast ADH1 gene (SONG and CARLSON 1998).

Manipulating Ras/PKA signaling activity in yeast cells: In this study, we used a variety of mutations and/or plasmids to influence Ras/PKA signaling activity. This section describes these reagents and their expected effects upon this pathway. All of these materials have been used previously in our lab to manipulate PKA activity in cells. To moderately increase Ras/PKA activity, the dominant, constitutively-active \( RAS2^{val19} \) allele was introduced into strains. This allele encodes a protein with diminished GTPase activity that is therefore found more often in its active GTP-bound form (TODA et al. 1985). Higher Ras/PKA activity was achieved by introducing an inducible allele of \( TPK1 \) that was under the control of the promoter from the copper-inducible \( CUP1 \) gene (DEMINOFF et al. 2006). To moderately down-regulate this pathway, a dominant-negative allele of \( RAS2, RAS2^{ala22} \), was introduced into cells (BUDOVSKAYA et al. 2002; POWERS et al. 1989). Both the \( RAS2^{val19} \) and \( RAS2^{ala22} \) alleles were under the control of the promoter from the inducible MET3 gene (MOUNTAIN et al. 1991). Expression from this promoter was induced by transferring cells to a medium that lacks methionine. For a more complete shutdown of Ras/PKA signaling, we used cells harboring an analog-sensitive allele of \( TPK1 \), referred to here as \( tpk1-as \), as the sole source of PKA activity (STEPHAN et al. 2009; YORIMITSU et al. 2007). This allele harbors an alteration within the active site that sensitizes the encoded protein to particular membrane inhibitors, like 1NM-PP1 (BISHOP et al. 2000; BISHOP et al. 1998). To inactivate PKA in this strain, we routinely exposed cells to
10 μM 1NM-PP1 for 4 hrs. PKA activity was also down-regulated by the introduction of a high-copy $PDE2$ plasmid; $PDE2$ encodes a high affinity cAMP phosphodiesterase (Sass et al. 1986).

**Western immunoblotting and immunoprecipitations:** Protein samples for Western blotting were prepared by a glass-beading method, separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare) as described (Budovskaya et al. 2002; Budovskaya et al. 2004). The membranes were then probed with the appropriate primary and secondary antibodies (GE Healthcare). The Supersignal chemiluminescent substrate (Pierce) was subsequently used to detect the reactive bands. Cell extracts for immunoprecipitation were prepared by resuspending cells in Lysis buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% Tween-20, 1 mM PMSF) and lysing by agitation with glass beads. HA-tagged proteins were immunoprecipitated with an anti-HA Sepharose matrix (Roche); extracts containing myc-tagged proteins were incubated with a monoclonal anti-myc antibody, and the immunoprecipitates were collected on Protein A sepharose (GE Healthcare). The amount of immunoprecipitated protein was assessed by Western blotting with the appropriate antibodies. The protein signals in the Western blots were quantified with the ImageJ image processing software program (Abramoff et al. 2004). Note that the protein samples in some cases were adjusted to obtain equal loading for the target protein of interest. This was necessary for those conditions that affected protein translation and thus resulted in lower amounts of total protein.

To monitor PKA phosphorylation in vivo, substrate proteins were precipitated under denaturing conditions as described with protease and phosphatase inhibitors present at all steps (Budovskaya et al. 2005; Deminoff et al. 2006; Herman et al. 1991). The level of PKA
phosphorylation was then assessed by Western blotting with the anti-PKA substrate antibody (Cell Signaling) as described (CHANG et al. 2004; DEMINOFF et al. 2006).

**In vitro kinases assays:** The immunoprecipitated substrate proteins were incubated with λ phosphatase (NEB) in λ phosphatase reaction buffer supplemented with 6 mM MnCl$_2$ for 1 hr and washed three times with wash buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% Tween-20, 1 mM PMSF). The *in vitro* kinase assay (IVKA) was then performed by incubating the immunoprecipitated material with 10 U of bPKA (Sigma) and 10 µCi [$\gamma$-$^{32}$P] ATP or 2.5 mM unlabeled ATP in a 40 µl reaction (50 mM potassium phosphate, 5 mM NaF, 10 mM MgCl$_2$, 4.5 mM DTT and both protease and phosphatase inhibitors). The proteins were separated by SDS-polyacrylamide gel electrophoresis and the level of phosphorylation was assessed by either autoradiography or Western blotting with the anti-PKA substrate antibody.

**Determination of the relative level of Ras2-GTP:** *E. coli* cells expressing the GST-Raf Ras-Binding Domain (RBD) fusion protein from plasmid pPHY2640 were grown in LB-Amp medium at 37°C to an OD$_{600}$ of 0.5/ml. Expression of the GST-Ras RBD was then induced by the addition of 100 µM IPTG for 3 hrs. Cells were then collected by centrifugation, resuspended in Lysis buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% Tween-20, 1 mM PMSF) and lysed by agitation with glass beads. The anti-GST antibody was added to the clarified cell extracts and the Raf RBD immunoprecipitates were collected on protein A-Sepharose beads. These beads, containing the Ras RBD, were washed three times with Wash buffer (25 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1% Tween-20, 1 mM PMSF) and incubated overnight at 4°C with the appropriate yeast cell extracts in Lysis buffer. The beads were then washed three times with
Lysis buffer, resuspended in SDS-urea sample buffer and the eluted proteins were separated by SDS-polyacrylamide electrophoresis. The amount of Ras2 protein present was then detected by Western blotting with an anti-Ras2 antibody (Santa Cruz).

**Spot growth assays:** Cells expressing the appropriate constructs were collected from mid-log phase cultures and diluted with water. The final concentration of cells was generally 5 OD\textsubscript{600} equivalents/ml. Assays were performed by spotting 5 μl of this cell suspension, and 5-fold serial dilutions thereafter, onto the appropriate growth medium, and incubating at 25° or 30°C for 2 to 3 days.

**RESULTS**

**The inhibition of TORC1 did not result in diminished levels of Ras/PKA signaling activity:** One of the main goals of this study was to test whether the inactivation of TORC1 would result in a concomitant decrease in Ras/PKA signaling activity. Therefore, we examined the effects of inactivating TORC1 with several reporters that examined both early and late steps in the Ras/PKA pathway. First, we assessed the level of PKA phosphorylation on two substrates, Srb9 and Rim15, with an anti-PKA substrate antibody. This antibody binds specifically to the PKA phosphorylated forms of these two proteins (Figure 1A, B; S1A, B) (CHANG et al. 2004). Srb9 is a component of the Mediator complex that is an essential co-activator for RNA polymerase II and Rim15 is a protein kinase required for the entry into a normal stationary phase (BORGGREFE et al. 2002; LIAO et al. 1995; REINDERS et al. 1998; SWINNEN et al. 2006). PKA phosphorylation has been shown to regulate the physiological activities of both these proteins (CHANG et al. 2004; REINDERS et al. 1998). For this analysis, Srb9 and Rim15 were immunoprecipitated from
cell extracts, and the relative level of phosphorylation was assessed by Western blotting with this α-PKA substrate antibody both before and after rapamycin treatment. Rapamycin is a specific inhibitor of the TORC1 signaling complex. We also examined an earlier step of the Ras/PKA signaling pathway by determining the relative amount of Ras2 present in its active, GTP-bound state after rapamycin treatment. The activated Ras2 was precipitated with a GST fusion protein containing the Ras Binding Domain (RBD) of the mammalian Raf-1 protein (RUDONI et al. 2001; TAYLOR and SHALLOWAY 1996). This Raf RBD binds specifically to the GTP-bound form of Ras2 (RUDONI et al. 2001; TAYLOR and SHALLOWAY 1996). The amount of Ras2 precipitated by this fusion was compared to the total Ras2 present in the input fraction by Western blotting with an antibody that recognizes both forms of the Ras2 protein.

We found that the PKA-dependent phosphorylation levels on Srb9 and Rim15 did not decrease upon rapamycin treatment (Figure 1A, B). No decrease was observed with either different concentrations of rapamycin or times of incubation (data not shown). To manipulate Ras/PKA signaling activity in a controlled fashion, we used a variety of pathway mutations and plasmids. A more detailed description of these reagents can be found in the Materials and Methods. For our purposes here, it is important to note that these manipulations resulted in the following levels of PKA activity, from the highest to lowest: the presence of an inducible TPK1, the presence of the constitutively-active RAS2val19 allele, wild-type cells, the presence of a dominant-negative RAS2ala22 allele, and the presence of a drug-sensitive tpk1-as allele. Each of the reporters used was found to be able to appropriately distinguish between these different levels of Ras/PKA signaling activity. Using these reagents, we also found that the levels of active GTP-bound Ras2 were not diminished following the inactivation of TORC1 (Figure 1C). In each experiment, the presence of rapamycin resulted in a significant decrease in the growth
rate of the treated cultures (see below). Altogether, these data were therefore inconsistent with models suggesting that the Ras/PKA pathway is a downstream effector of the TORC1 complex.

**Inactivation of Ras/PKA signaling did not result in a loss of TORC1 activity:** To also assess how the Ras/PKA pathway might influence TORC1 activity, we took advantage of recent studies indicating that Atg13 is phosphorylated *in vivo* by both PKA and TORC1 (Kamada *et al.* 2010; Stephan *et al.* 2009). These phosphorylation events appear to occur at distinct sites that can be monitored separately. Therefore, Atg13 can be used to simultaneously report on the activities of both of these signaling pathways. The level of PKA phosphorylation can be assessed with the anti-PKA substrate antibody as described above (Figure 2A; S1C). Using this assay, we found that the extent of PKA phosphorylation on Atg13 did not decrease upon rapamycin treatment (Figure 2A). Instead, we detected a slight, but consistent, elevation in this signal upon TORC1 inactivation (Figure 2A). A similar increase in PKA phosphorylation following rapamycin treatment was also observed with both Srb9 and Rim15 (see Figure 1A, B).

The relative level of TORC1 phosphorylation on Atg13, on the other hand, can be inferred from the anomalous migration pattern observed in SDS-polyacrylamide gels. TORC1-dependent phosphorylation causes this protein to migrate as a broad smear on these gels (Figure 2B) (Kamada *et al.* 2000; Scott *et al.* 2000). Upon rapamycin treatment, this smear collapses into a tight, faster-migrating band (Figure 2B). Moreover, conditions that are thought to increase TORC1 activity resulted in an “upward” shift of this Atg13 smear. For example, this shift up was noted in cells treated with cycloheximide and expressing higher levels of Tor1 (Figure 2B; S1D). Recent studies have suggested that TORC1 activity is elevated upon cycloheximide treatment and the over-expression of Tor1 likely results in elevated TORC1 activity because this
protein is associated exclusively with TORC1 (Beugnet et al. 2003; Loewith et al. 2002; Urban et al. 2007). Therefore, the relative degree of Atg13 retardation in an SDS-polyacrylamide gel can be used to assess the level of TORC1 activity in cells. We used this assay here to examine how fluctuations in Ras/PKA activity might influence TORC1 signaling. Interestingly, we found that there again appeared to be an antagonistic relationship between these pathways. Decreased levels of PKA activity resulted in an upward shift of the Atg13 smear whereas elevated PKA activity caused a partial collapse into a less phosphorylated form (Figure 2C). In all, these results with Atg13 suggested that both the Ras/PKA and TORC1 pathways, through either direct or indirect means, might somehow restrain the activity of the other pathway.

**Decreased TORC1 signaling resulted in elevated levels of Cki1 phosphorylation by PKA:**

To examine further the effects of TORC1 signaling activity on the Ras/PKA pathway, we employed an additional reporter of PKA activity, the choline kinase, Cki1. Cki1 activity is important for the biosynthesis of the membrane phospholipid, phosphatidylcholine (Kim et al. 1998). Cki1 has been shown to be phosphorylated by PKA at two sites near the N-terminus, Ser-30 and Ser-85 (Kim and Carmán 1999; Yu et al. 2002) (Figure 3A). A third position, Ser-25, appears to be recognized by protein kinase C (Choi et al. 2005). We recently found that an epitope-tagged fragment of Cki1, containing the N-terminal 200 residues of this protein, migrated as a doublet in SDS-polyacrylamide gels (Figure 3A, B) (Deminoff et al. 2006). The presence of the slower-migrating band was lost upon phosphatase treatment and was restored by a subsequent incubation with PKA and ATP (Figure 3B). These results suggested that the presence of the upper band, referred to here as Cki1-P*, was the result of PKA phosphorylation.
To map the site responsible for the presence of Cki1-P*, we sequentially replaced the three serine residues indicated above with an alanine, and examined the mobility of the altered proteins on SDS-polyacrylamide gels. This analysis indicated that the third serine, Ser-85, was necessary and sufficient for the observed mobility shift (Figure 3C). This assertion was supported by results from *in vitro* kinase assays where radioactivity was incorporated into only the slower-migrating form of the Cki1-AAS variant but into both forms of the Cki1-SSS fragment (Figure 3D). Finally, as with the wild-type Cki1 fragment, the slower-migrating form of the Cki1-AAS variant was absent following phosphatase treatment and was regenerated in an *in vitro* kinase reaction with PKA (Figure 3B). In all, these data indicated that PKA phosphorylation of Ser-85 altered the mobility of this Cki1 fragment on SDS-polyacrylamide gels.

To test whether this Cki1 fragment could be used as a reporter for *in vivo* PKA activity, we examined how the level of Cki1-P* was affected by variations in Ras/PKA signaling. Using the reagents described above, we found that the relative amount of Cki1-P* accurately reflected the levels of Ras/PKA signaling activity present in cells (Figure 3E, F; S2A). These results indicated that Cki1 was phosphorylated *in vivo*, as well as *in vitro*, by PKA. In addition, this assay did not require an additional immunoprecipitation step prior to the assessment of the PKA phosphorylation level. We therefore used this reporter to assess the consequences of inactivating TORC1 activity with rapamycin.

Interestingly, we found that rapamycin treatment did not lead to a decrease in the relative amount of Cki1-P* (Figure 4A). Instead, we reproducibly observed an increase in the amount of Cki1-P* suggesting that PKA activity might increase following the inactivation of TORC1 (Figure 4A; S2B). This increase in Cki1-P* was not observed in cells where growth or division
had been arrested by other means. For example, no increase in the relative amount of Cki1-P* was detected in cells treated with the protein synthesis inhibitor, cycloheximide (Figure S2B). In addition, the level of Cki1-P* was not elevated in *prt1-1* or *cdc28-1* mutants that had been transferred to a non-permissive temperature for periods varying from one to eight hours (Figure 4B; S3A, B; data not shown). In *S. cerevisiae*, *PRT1* encodes a subunit of the eukaryotic translation initiation factor, eIF3, and *CDC28* encodes a cyclin-dependent kinase required for progression through the cell cycle (LORINCZ and REED 1984; NARANDA *et al.* 1994). These latter results suggested that the increase in Ras/PKA activity observed here might be a specific response to diminished Tor signaling.

Finally, we examined the timing of the increase in Cki1 phosphorylation with respect to the rapamycin effects on cell growth. In these studies, we found that the increase in Cki1-P* was detectable about 30 to 45 minutes after the addition of rapamycin, and that this occurred just before the cells exhibited a notable decrease in growth rate (Figure 4C, D; S2B). In general, the cultures treated with rapamycin did not show a significant slow down of growth until more than 1 hr after the addition of this drug (Figure 4C). These rapamycin-induced increases in phosphorylation were dependent upon the presence of PKA activity as they were not observed in *tpk1-as* cells that had been treated with the drug, 1NM-PP1 (Figure S3C). In all, these studies with Cki1 were therefore consistent with those above and suggested that the Ras/PKA pathway was not positively regulated by TORC1 activity. Instead, these data again suggested that Ras/PKA signaling activity might increase in response to the inactivation of TORC1.

**Diminished PKA signaling resulted in an enhanced resistance to rapamycin:** To further examine the relationship between the PKA and TORC1 pathways, we assessed how altering
PKA activity might influence the response to rapamycin. Interestingly, these genetic results were consistent with the relationship suggested by the above studies with the molecular reporters for each pathway. In particular, we found that cells with diminished Ras/PKA signaling exhibited an elevated resistance to rapamycin. For example, cells containing a temperature-sensitive (ts) Ras2 protein as the only source of Ras activity were relatively more resistant to rapamycin than wild-type cells when grown at semi-permissive temperatures (Figure 5A; S4). This rapamycin resistance was not observed with other ts strains, like prt1-1 and cdc28-1 mutants, that were also impaired for cell growth and/or division (Figure 5B) (HANIC-JOYCE et al. 1987; HARTWELL 1978). Finally, cells lacking the inhibitory subunit of PKA, Bcy1, were significantly more sensitive to rapamycin than the wild-type; bcy1Δ cells have constitutively elevated levels of PKA activity (Figure 5C) (TODA et al. 1987a; WERNER-WASHBURN et al. 1993). Therefore, the level of rapamycin resistance appeared to be inversely correlated with Ras/PKA signaling activity.

These rapamycin effects were examined further with other means of manipulating PKA activity and in additional genetic backgrounds. For example, we also tested the consequences of introducing the RAS2val19 allele and a high-copy PDE2 plasmid. The over-expression of Pde2, a high-affinity cAMP phosphodiesterase, leads to decreased levels of PKA activity (HOWARD et al. 2002; HOWARD et al. 2003; SASS et al. 1986). Consistent with the above results, we found that the presence of RAS2val19 resulted in an increased sensitivity to rapamycin whereas the elevated levels of Pde2 were associated with an elevated resistance to this drug (Figure 6A). Similar results were observed with multiple lab strains, including those in the W303, SEY6210 and BY genetic backgrounds (Figure 6A-C). It is important to point out that different effects have been reported for one particular strain background. These studies found that increased Ras/PKA
signaling in particular Σ (Sigma) strains resulted in an elevated resistance to rapamycin (ZURITAMARTINEZ and CARDEÑAS 2005). The underlying reasons for these different effects are not known and will require additional study. In all, however, the data here suggested that decreased Ras/PKA activity in most lab strains was associated with an increased resistance to rapamycin and vice-versa.

**Genetic interactions between Ras/PKA and Tor signaling pathway mutations:** The above data indicated that decreased Ras/PKA activity resulted in an elevated resistance to rapamycin. This increased resistance could have been due to the presence of elevated TORC1 activity (see Figure 2C) or to effects on other aspects of cell physiology that influence the response to rapamycin, including the general uptake of this drug. We therefore examined the genetic interactions occurring between Ras/PKA and Tor pathway mutations. In particular, we asked how altering Ras/PKA signaling would impact the growth of strains with diminished Tor activities. For this analysis, we used strains that were defective in TORC1- and potentially TORC2-related activities (HELLIWELL et al. 1998; REINKE et al. 2004). Interestingly, the presence of the \( \text{RAS2val19} \) allele was found to exacerbate the growth defects associated with these \( \text{tor1Δ}, \text{tor1Δ tor2ts} \) and \( \text{tor2ts} \) strains (Figure 7A, B). In addition, the temperature-sensitive growth of the latter strain was suppressed by the over-expression of Pde2 (Figure 7B). Therefore, these results are consistent with the above effects with rapamycin and taken together suggested that the Ras/PKA pathway might be influencing Tor signaling activities in *S. cerevisiae* cells.
PKA phosphorylation levels of multiple substrates were elevated in response to nitrogen deprivation: We were interested in whether the response of the PKA pathway to rapamycin that was observed here was a reflection of normal processes occurring in *S. cerevisiae* cells. To begin to address this question, we asked how the PKA pathway would respond to changes in the nitrogen levels present in the growth medium. Previous studies have suggested that the TORC1 pathway is responding to nitrogen levels in the environment, although the precise upstream signal has not yet been definitively identified (De Virgilio and Loewith 2006a; Dechant and Peter 2008). For these experiments, we examined three of the Ras/PKA reporters used above, Atg13, Cki1 and Srb9. Atg13 was especially valuable here because this protein could also report on the levels of TORC1 activity present. As noted previously, we found that the transfer of cells to a nitrogen-limiting medium resulted in a collapse of the Atg13 smear, indicative of decreased TORC1 signaling in these cells (Kamada et al. 2000; Scott et al. 2000). In contrast, the level of PKA-dependent phosphorylation on each of the reporters was found to increase following nitrogen deprivation (Figure 8A-C). These results largely mirrored those obtained above with rapamycin although the magnitude of the change tended to be greater in the nitrogen-limiting conditions. These effects also differed tremendously from those observed upon carbon source deprivation where the PKA signal on both Cki1 and Srb9 was dramatically reduced (Figure 8B, C). In all, these data therefore suggested that Ras/PKA signaling might increase upon nitrogen limitation, a condition that would be expected to result in diminished TORC1 activity.
DISCUSSION

The cellular response to environmental signals can be modulated by interactions occurring between components of different signal transduction pathways. In this study, we set out to characterize any such interactions occurring between the Tor and Ras/PKA signaling pathways in the yeast, *S. cerevisiae*. These pathways are known to control a similar set of biological processes important for growth in this yeast (DECHANT and PETER 2008; ZAMAN et al. 2008). However, despite these similarities, our data suggest that a mutual antagonism exists between these pathways. In particular, we found that the inactivation of either pathway resulted in elevated activity through the other. For example, decreased TORC1 signaling was found to result in elevated levels of PKA activity. This was demonstrated with multiple reporters that assessed both early and late stages of the Ras/PKA signaling pathway. The increases were reproducible and observed in a number of different strain backgrounds. Conversely, decreased PKA signaling was found to result in a similar increase in the TORC1-dependent phosphorylation of at least one reporter, Atg13. Importantly, we also detected genetic interactions that were consistent with these molecular results. For example, increased Ras/PKA activity exacerbated the growth defects associated with both rapamycin treatment and mutants with diminished Tor signaling. A similar genetic interaction was detected previously with a conditional allele of *KOG1*; *KOG1* encodes an essential component of TORC1 that is analogous to the mammalian Raptor protein (ARAKI et al. 2005; LOEWITH et al. 2002; WEDAMAN et al. 2003). The growth defects associated with this *kog1* allele were found to be suppressed by the presence of a high-copy *PDE2* plasmid (ARAKI et al. 2005). In addition, a recent microarray study suggested that Sch9 might negatively regulate aspects of Gpa2/PKA signaling; Sch9 is a downstream effector of TORC1 (URBAN et al. 2007; ZAMAN et al. 2009). Altogether, these data
suggest that limiting the activity of either the PKA or Tor pathway results in increased signaling through the other.

In all, our results are most consistent with a model where the Ras/PKA and Tor pathways function in parallel to control cell growth. In no instance did we find that the inactivation of one pathway was associated with a concomitant decrease in the other. However, our data clearly indicate that these pathways do exert some level of negative control over the other. A key question is why such antagonism might exist between signaling pathways that are both thought to promote cell growth. One intriguing possibility is that these interactions could provide a buffering capacity to the cell that would allow for a more constant rate of growth in the face of changing environmental conditions. In this model, conditions that result in diminished TORC1 activity would not immediately affect growth because of the compensatory increase in Ras/PKA signaling and vice versa. This type of regulatory circuitry could ensure that the cell does not prematurely arrest growth in response to relatively minor changes in the environment. As such, this buffering could provide a growth advantage over cells that might otherwise begin to unnecessarily lapse into a growth-arrested state. However, this buffering would presumably have its limits as a significant loss of signaling through either the TORC1 or PKA pathway is known to lead to a stationary phase-like growth arrest (ZAMAN et al. 2008).

A separate question concerns the underlying mechanism(s) responsible for the interactions observed here. In particular, we would like to know whether this potential crosstalk is the result of direct interactions between components of these two pathways or is due to something more indirect. Although the changes in Cki1 phosphorylation were found to occur before the rapamycin-mediated effects on growth, many of the consequences of rapamycin treatment have been shown to occur with more rapid kinetics (DE VIRGILIO and LOEWITH 2006b;
Therefore, the effects on the PKA pathway observed here could be an indirect consequence of the inactivation of TORC1. One possibility is that the growth inhibitory effects of rapamycin could result in the production of a signal that stimulates the activity of the Ras/PKA pathway. However, it is important to point out that this kinetic argument does not preclude the possibility of a direct interaction between these two pathways. Different substrates could be dephosphorylated at different rates and this could result in both immediate and more delayed responses to the loss of TORC1 activity. It should also be emphasized that the observed increase in Ras/PKA signaling was not generally associated with a slow down of cell growth and appeared to be a more specific response to limiting Tor activity. Finally, our data suggest that the inactivation of TORC1 might be affecting a relatively early step in the Ras/PKA pathway. This assertion follows from the observation that the relative level of active, or GTP-bound, Ras2 was elevated in response to rapamycin (Figure 1). Thus, the loss of TORC1 signaling appears to influence either this, or an earlier, step of the Ras/PKA pathway. Future work will be directed at identifying this target and the nature of the signal that elicits this response.

It ultimately will be important to demonstrate that the potential buffering proposed above is indeed occurring under normal physiological conditions. Our results here with nitrogen limitation may represent an important step toward this goal. In particular, we found that the PKA-dependent phosphorylation of multiple substrates was elevated in response to nitrogen deprivation. This result is interesting as the TORC1 pathway is thought to be controlled, at least in part, by the levels and/or quality of the available nitrogen source (Dechant and Peter 2008; Wullschleger et al. 2006; Zaman et al. 2008). Therefore, the increased PKA activity detected may have been due to the lower levels of TORC1 signaling in these cells. The ability to buffer
the response to environmental change is an important characteristic of many biological processes from individual metabolic pathways to animal development (HARTMAN et al. 2001; RUTHERFORD 2000; WADDINGTON 1942). This capacity provides a robustness that ensures a more predictable outcome in the face of external (or internal) perturbations. We suggest that the potential antagonism between the PKA and Tor pathways described here could serve this sort of a function during the control of S. cerevisiae growth.

ACKNOWLEDGEMENTS

We thank James Broach, Marian Carlson, Michael Hall, Michael Hampsey, David Shalloway and Ted Powers for reagents used in this study, and members of the Herman lab, and especially Stephen Deminoff, for comments on the manuscript. This work was supported by a grant from the National Institutes of Health (GM65227) to P.K.H.
LITERATURE CITED

ABRAMOFF, M. D., P. J. MAGELHAES and S. J. RAM, 2004 Image processing with ImageJ. Biophotonics International 11: 36-42.

ARAKI, T., Y. UESONO, T. OGUCHI and E. A. TOH, 2005 LAS24/KOG1, a component of the TOR complex 1 (TORC1), is needed for resistance to local anesthetic tetracaine and normal distribution of actin cytoskeleton in yeast. Genes Genet Syst 80: 325-343.


KAMADA, Y., K. YOSHINO, C. KONDO, T. KAWAMATA, N. OSHIRO et al., 2010 Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol Cell Biol 30: 1049-1058.


Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog

LIAO, S. M., J. ZHANG, D. A. JEFFERY, A. J. KOLESKE, C. M. THOMPSON et al., 1995

LIPPMAN, S. I., and J. R. BROACH, 2009
Protein kinase A and TORC1 activate genes for ribosomal biogenesis by inactivating repressors encoded by Dot6 and its homolog Tod6.

LOEWITH, R., E. JACINTO, S. WULLSCHLEGER, A. LORBERG, J. L. CRESPO et al., 2002
Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 10: 457-468.

LORINCZ, A. T., and S. I. REED, 1984

MBONYI, K., M. BEULLENS, K. DETREMERIE, L. GEERTS and J. M. THEVELEIN, 1988
Requirement of one functional RAS gene and inability of an oncogenic ras variant to mediate the glucose-induced cyclic AMP signal in the yeast Saccharomyces cerevisiae. Mol Cell Biol 8: 3051-3057.


NARANDA, T., S. E. MACMILLAN and J. W. HERSHEY, 1994
PEDRUZZI, I., F. DUBOULOZ, E. CAMERONI, V. WANKE, J. ROOSEN et al., 2003 TOR and PKA Signaling Pathways Converge on the Protein Kinase Rim15 to Control Entry into G(0). Mol Cell 12: 1607-1613.


REINKE, A., S. ANDERSON, J. M. MCCAFFERY, J. YATES, 3RD, S. ARONOVA et al., 2004 TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. J Biol Chem 279: 14752-14762.


FIGURE LEGENDS

FIGURE 1. Assessing the effects of rapamycin on reporters of Ras/PKA signaling activity. (A) The PKA phosphorylation of Srb9 did not decrease upon rapamycin treatment. The relative level of PKA phosphorylation on Srb9 was assessed by Western blotting with an anti-PKA substrate antibody (α-Sub), as described in the Materials and Methods. This antibody specifically recognizes a PKA phosphorylated form of Srb9 (Chang et al. 2004). Ras/PKA signaling levels were up- or down-regulated by either inducing expression from the CUP1-TPK1 construct (Tpk1) or inactivating the tpk1-as allele with the drug, 1NM-PP1, respectively. Rapamycin was added to a final concentration of 200 ng/ml where indicated (Rap), and the relative levels of Srb9 phosphorylation were assessed after 2 hrs of incubation. The parenthetical notation below each panel indicates the effects that the experimental conditions had on PKA or TORC1 signaling activity. (B) The extent of PKA phosphorylation on Rim15 was not diminished upon the inactivation of TORC1. The relative level of PKA phosphorylation was assessed by Western blotting with the anti-PKA substrate antibody (α-Sub). Left, The Rim15 fragment was precipitated from yeast cell extracts and then treated with λ phosphatase. The sample was washed and split into two aliquots. One aliquot was mock treated (-PKA) whereas the second was subjected to an in vitro kinase reaction with bPKA and 2.5 mM ATP (+PKA). Middle, The relative level of PKA phosphorylation was assessed in cells containing inducible forms of either the dominant-negative allele, Ras2ala22, or the dominant-positive, Ras2val19. Expression of each construct was induced from the MET3 promoter by transferring cells to a methionine-free medium for 2 hr. Vec, vector control. Right, Rapamycin was added at a final concentration of 200 ng/ml for 2 hr before assessing the relative amount of PKA phosphorylation on the Rim15 reporter. (C) Assessing the effects of rapamycin on the levels of active, GTP-
bound Ras2. The levels of Ras2-GTP were assessed as described in the Materials and Methods. Briefly, a GST fusion protein containing the Ras Binding Domain of the mammalian Raf-1 protein (GST-Raf) was used to precipitate the active, GTP-bound Ras2 from cell extracts. The extracts were prepared from cells that had been treated with 0 or 200 ng/ml rapamycin for 2 hr. The amount of precipitated Ras2 protein was then assessed by Western blotting with an anti-Ras2 antibody (Ras2-GTP). The Input panel shows the total amount of Ras2 present in each cell extract (Total Ras2).

FIGURE 2. Atg13 as a reporter for both Ras/PKA and TORC1 signaling activity. (A) Decreased TORC1 signaling resulted in elevated levels of PKA phosphorylation on Atg13. The PKA phosphorylation level of Atg13 was assessed by Western blotting with the anti-PKA substrate antibody ($\alpha$-Sub). Controls indicating that this Atg13 signal was responsive to Ras/PKA signaling levels in the cell are shown. Decreased Ras/PKA signaling was achieved by addition of the inhibitor, 1NM-PP1, to the tpkl-as strain or expression of an inducible dominant-negative $RAS2^{ala22}$ construct. Elevated Ras/PKA activity was achieved by the expression of an inducible dominant-positive allele of $RAS2$, $RAS2^{val19}$. The notation beneath each panel indicates the expected effects on TORC1 signaling activity. (B) The anomalous migration of Atg13 on SDS-polyacrylamide gels can be used as an in vivo measure of TORC1 signaling activity. The extent of the Atg13 “smear” was assessed in cells that had been treated with 200 ng/ml rapamycin (Rap) or 50 µg/ml cycloheximide (CHX) for 1 hr by Western blotting with an anti-myc antibody. Note that the extent of the Atg13 smear is dependent upon the running conditions of the gel. For example, a shorter separation time was used to obtain the tighter bands in panel A. (C) The relative level of the TORC1-dependent phosphorylation of Atg13 was inversely
proportional to Ras/PKA signaling levels. The extent of the Atg13 smear was assessed by Western blotting with an antibody specific for the HA epitope. Elevated Ras/PKA signaling was achieved by over-expression of an HA epitope-tagged Tpk1 (Tpk1-HA) and decreased signaling by the expression of an inducible dominant-negative \( RAS2^{ala22} \) construct.

FIGURE 3. Cki1 phosphorylation can be used to report on the \textit{in vivo} levels of Ras/PKA signaling activity. (A) A schematic of the Cki1 protein showing the positions of the two serine residues recognized by PKA, Ser-30 and Ser-85, and the choline kinase domain (light gray shading). The position of the putative PKC site, Ser-25, is also shown. The portion of the Cki1 protein expressed in the Cki1 reporter fragment used here is shown with dark gray shading. The asterisk indicates the PKA site, Ser-85, responsible for the anomalous migration of the Cki1 reporter construct. (B) The altered mobility of Cki1 on SDS-polyacrylamide gels was the result of PKA phosphorylation. The indicated versions of the Cki1 fragment were precipitated from yeast cell extracts and then treated with \( \lambda \) phosphatase, as indicated. The fragments were washed and then incubated with bovine PKA (bPKA) and 2.5 mM ATP, as described in the Materials and Methods. Cki1-P* refers to the PKA phosphorylated form of the Cki1 fragment that exhibited an altered mobility on gels. PPase, \( \lambda \) phosphatase. (C) The presence of Ser-85 was necessary and sufficient for the Cki1 mobility shift \textit{in vivo}. Extracts were prepared from yeast cells expressing the indicated Cki1 variants, and the gel mobility of each variant was assessed by Western blotting with an anti-myc antibody. (D) Ser-85 phosphorylation by PKA was responsible for the slower-migrating form of Cki1. The indicated Cki1 variants were immunoprecipitated from cell extracts and either mock treated (-) or incubated with bPKA and \([\gamma-^{32}\text{P}] \) ATP. The reaction products were then separated on SDS-polyacrylamide gels and the
level of PKA phosphorylation was assessed by autoradiography ($^{32}$P). The relative amount of the variants present in each reaction was assessed by Western blotting with an anti-myc antibody. (E) Elevated Ras/PKA signaling resulted in an increase in the relative levels of Cki1-P*. The relative level of Cki1-P* was assessed in cells containing either a TPK1, RAS2$^{val19}$ or vector plasmid by Western blotting with an anti-myc antibody. The TPK1 gene was over-expressed from the copper-inducible CUP1 promoter. (F) Inactivation of Ras/PKA signaling resulted in diminished levels of Cki1-P*. The Cki1 fragment was expressed in a yeast strain, Y3175, that had an analog-sensitive allele of TPK1 as the sole source of PKA activity. This Tpk1 variant was inactivated by the addition of the inhibitor, 1NM-PP1 (1NM). The relative level of Cki1-P* was assessed after 4 hrs of incubation with 10 μM 1NM-PP1.

FIGURE 4. Inactivation of TORC1 signaling resulted in an increase in the PKA phosphorylation of Cki1. (A) TORC1 inactivation by rapamycin caused an increase in the relative levels of Cki1-P*. The relative levels of Cki1-P* were assessed in cell extracts after a 2 hr incubation with 20 (Lo) or 200 (Hi) ng/ml of rapamycin. (B) The relative levels of Cki1-P* were not elevated in prt1- or cdc28-arrested cells. Wild-type, prt1-1 and cdc28-1 cells were grown to mid-log phase at 25°C and then shifted to the non-permissive temperature of 39°C for 8 hr. Cell extracts were prepared and the relative levels of Cki1-P* were assessed by Western blotting. The strains analyzed were PHY1682 (Wild-type, Wt), PHY1086 (prt1) and PHY1235 (cdc28). (C) The relative increase in Cki1-P* levels was coincident with the rapamycin-mediated inhibition of growth. The kinetics of growth inhibition by rapamycin. A growth curve showing cell density in cultures treated with either 0 (-R) or 200 (+R) ng/ml rapamycin. The point of rapamycin addition ($t = 0$) is indicated by the arrow. (D) A Western blot showing the relative levels of
Cki1-P* in the same culture at the indicated times after the addition of 200 ng/ml rapamycin. The 3(-R) sample shows the level of Cki1-P* in the control culture that was incubated for 3 hrs in the absence of rapamycin. The relative amount of Cki1 present in each band was assessed with the ImageJ software program and the Cki1-P*/Cki1 ratio is shown for each time-point.

FIGURE 5. Diminished Ras/PKA signaling was associated with an increased resistance to rapamycin. (A) The temperature-sensitive ras2-23 strain exhibited an increased resistance to rapamycin at a semi-permissive temperature. Equal amounts of log phase cells of the indicated genotypes were collected, diluted in water and spotted onto YPAD plates in a series of five-fold dilutions. The plates were incubated at the permissive (25°C) or semi-permissive (30°C) temperature for 2 to 3 days with the indicated concentrations of rapamycin. The strains analyzed were PHY1682 (Wild-type), PHY1120 (ras1 RAS2) and PHY1150 (ras1 ras2-23). (B) The effects of rapamycin on the growth of prt1-1 and cdc28-1 mutants. Aliquots of the indicated strains were plated as described above and incubated for 2 to 3 days at the permissive (25°C) or semi-permissive (30°C) temperature with the indicated concentration of rapamycin. The strains analyzed were PHY1682 (Wild-type), PHY1086 (prt1-1) and PHY1235 (cdc28-1). (C) Yeast bcy1Δ strains exhibited a heightened sensitivity to rapamycin. Aliquots of the indicated strains were plated as described above and incubated for 2 to 3 days at 30°C. The concentration of rapamycin present is indicated at the right hand side of the images.

FIGURE 6. The inverse relationship between Ras/PKA signaling activity and the resistance to rapamycin was observed in multiple genetic backgrounds. In panels A-C, log phase cells were collected, diluted in water and plated onto a minimal medium containing the indicated amounts
of rapamycin. Each column represents a five-fold serial dilution of the sample to the immediate left. The plates were then incubated for 2 to 3 days at 30°C before imaging. Each strain contained a single-copy \( RAS2^{val19} \) plasmid, a high-copy \( PDE2 \) construct and a vector control. The genetic backgrounds of each of the tested strains are indicated: PHY1682 (W303 background), PHY1220 (SEY6210) and BY4741 (BY).

FIGURE 7. The growth defects associated with Tor pathway mutations were influenced by altered Ras/PKA signaling activity. (A) The presence of \( RAS2^{val19} \) exacerbated the slow growth phenotype of \( tor1\Delta \) strains. Wild type, \( tor1\Delta \) and \( tor1\Delta tor2^{ts} \) cells containing either a vector or \( RAS2^{val19} \) plasmid were grown to log phase and spotted onto YM-glucose plates. The plates were incubated at 37°C for 2 to 3 days before imaging. The strains examined were JK9-3da (Wild-type), PLY297 (\( tor1\Delta \)) and NB4-6a (\( tor1\Delta tor2-21^{ts} \)). (B) The temperature-sensitive growth defects of a \( tor2^{ts} \) strain were suppressed by the over-expression of the Pde2 cAMP phosphodiesterase and exaggerated by the presence of \( RAS2^{val19} \). Wild-type (SH100) and \( tor2^{ts} \) (SH121) cells carrying the indicated plasmids were grown to log phase and serial dilutions of these cultures were plated to YM-glucose plates. The plates were then incubated at the indicated temperatures for 2 to 3 days before examination.

FIGURE 8. PKA activity \textit{in vivo} was elevated in response to nitrogen starvation. (A) Nitrogen deprivation had opposing effects upon the PKA- and TORC1-dependent phosphorylation of Atg13. Yeast cells were grown to mid-log phase in SC-glucose minimal medium (SC) and then transferred to the nitrogen starvation medium, SD-N (-N), for 3 hrs. The relative levels of the PKA- and TORC1-dependent phosphorylation on Atg13 were assessed by Western blotting with
the anti-PKA substrate (α-Sub) or anti-HA (α-HA) antibodies, respectively. The Atg13 was resolved further to achieve the separation observed in the bottom panel. (B) The relative level of Cki1-P* was elevated in response to nitrogen deprivation. Cells containing the Cki1 reporter fragment were grown to mid-log phase in SC-glucose minimal medium and then transferred to a SC medium lacking glucose for 15 mins or SD-N medium for 3 hrs. The relative levels of Cki1-P* were then determined by Western blotting. Note that the levels of Cki1-P* were dramatically reduced upon carbon starvation. (C) The level of PKA phosphorylation on Srb9 was elevated in response to nitrogen limitation. The nitrogen and carbon starvations were carried out as described in B, except that the incubation in the SC medium lacking glucose was 60 mins. The Srb9 was then precipitated from these cell extracts and the extent of PKA phosphorylation was assessed by Western blotting with the anti-PKA substrate antibody (α-Sub).
TABLE 1. The yeast strains used in this study.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>REFERENCE/SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK9-3da</td>
<td>MATα leu2-3,112 ura3-52 rme1 trp1 his4 GAL+ HMLα</td>
<td>(KUNZ et al. 1993)</td>
</tr>
<tr>
<td>NB4-6a</td>
<td>MATa leu2-3,112 ura3-52 rme1 trp1 his4 GAL+ HMLα</td>
<td>Dr. Michael Hall</td>
</tr>
<tr>
<td></td>
<td>tor1::HIS3 tor2::ADE2 pNB21 (YEplac181::tor2-21)</td>
<td></td>
</tr>
<tr>
<td>PHY1025</td>
<td>MATα his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>lys2Δ:: hisG</td>
<td></td>
</tr>
<tr>
<td>PHY1086</td>
<td>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>prt1-1</td>
<td></td>
</tr>
<tr>
<td>PHY1120</td>
<td>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>ras1Δ::HIS3</td>
<td></td>
</tr>
<tr>
<td>PHY1150</td>
<td>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>ras1Δ::HIS3 ras2-23</td>
<td></td>
</tr>
<tr>
<td>PHY1160</td>
<td>MATα ADE2 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>lys2Δ:: hisG bey1Δ::LEU2</td>
<td></td>
</tr>
<tr>
<td>PHY1220</td>
<td>MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52</td>
<td>(CHANG et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>suc2-39</td>
<td></td>
</tr>
<tr>
<td>PHY1235</td>
<td>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</td>
<td>(HERMAN and RINE 1997)</td>
</tr>
<tr>
<td></td>
<td>cdc28-1</td>
<td></td>
</tr>
<tr>
<td>PHY1682</td>
<td>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</td>
<td></td>
</tr>
<tr>
<td>PHY4231</td>
<td>MATα hisΔ 1 leu2Δ0 met15Δ0 ura3Δ0 (BY4741)</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td>PJ69-4A</td>
<td>MATα trp 1-901 leu2-3,112 ura3-52 his3-200 gal4Δ</td>
<td>(JAMES et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>gal180Δ GAL2-ADE2 LYS2::GAL1-HIS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>met2::GAL7-lacZ</td>
<td></td>
</tr>
<tr>
<td>PLY297</td>
<td>MATα leu2-3, 112 trp1 ura3 rme1 his4 tor1::TRP1</td>
<td>(REINKE et al. 2004)</td>
</tr>
<tr>
<td>SH100</td>
<td>MATα leu2-3,112 ura3-52 rme1 trp1 his4 HMLα ade2Δ</td>
<td>(HELLIWELL et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>tor2Δ::ade2-3 YCplac111:: TOR2</td>
<td></td>
</tr>
</tbody>
</table>
SH121  \(\text{MAT}a \text{ leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2}\Delta\)  
\(\text{tor2}\Delta::\text{ade2-3 YCplac111:: tor2-21}\)  
(HELLIWELL et al. 1998)

Y3175  \(\text{MAT}a \text{ ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1}\)  
\(\text{ura3-1 tpk2::KAN tpk3::TRP1 tpk1(M164G)}\)  
Dr. James Broach
Figure 2 - Ramachandran et al, 2010

A

<table>
<thead>
<tr>
<th></th>
<th>1NM-PP1</th>
<th>RAS2^ala22</th>
<th>RAS2^val19</th>
<th>Rap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atg13</td>
<td>-</td>
<td>(↓ PKA)</td>
<td>(↓ PKA)</td>
<td>(↓ TORC1)</td>
</tr>
<tr>
<td>Atg13</td>
<td>+</td>
<td>(↑ PKA)</td>
<td>(↑ PKA)</td>
<td>(↓ TORC1)</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Rap</th>
<th>CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atg13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(↓ TORC1)</td>
<td>(↑ TORC1)</td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>Tpk1-HA</th>
<th>RAS2^ala22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atg13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(↑ PKA)</td>
<td>(↓ PKA)</td>
<td></td>
</tr>
<tr>
<td>Tpk1-HA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4 - Ramachandran et al, 2010

A

Rap

-  Hi  Lo

Cki1-P*  Cki1

B

Wt  prt1  Wt  cdc28

Cki1-P*  Cki1

C

Culture density (OD_{600})

-2  0  1  2  3

Time (hrs)

D

Time (hr):

0  0.5  1  1.5  2  2.5  3

Cki1-P*  Cki1

0.32  0.64  1.22  1.30  1.53  3.26  2.14  0.62  0.32
Figure 8 - Ramachandran et al, 2010

A

SC   -N

Atg13 -

\( \alpha \)-Sub

Atg13 -

\( \alpha \)-HA

Atg13-P

\( \alpha \)-HA

B

SC   -C   -N

Cki1-P*

Cki1

C

-N   SC   -C

Srb9 -

\( \alpha \)-Sub

Srb9 -

\( \alpha \)-HA