Caffeine extends yeast lifespan by targeting TORC1

WANKE, Valeria, et al.

Abstract
Dietary nutrient limitation (dietary restriction) is known to increase lifespan in a variety of organisms. Although the molecular events that couple dietary restriction to increased lifespan are not clear, studies of the model eukaryote Saccharomyces cerevisiae have implicated several nutrient-sensitive kinases, including the target of rapamycin complex 1 (TORC1), Sch9, protein kinase A (PKA) and Rim15. We have recently demonstrated that TORC1 activates Sch9 by direct phosphorylation. We now show that Sch9 inhibits Rim15 also by direct phosphorylation. Treatment of yeast cells with the specific TORC1 inhibitor rapamycin or caffeine releases Rim15 from TORC1-Sch9-mediated inhibition and consequently increases lifespan. This kinase cascade appears to have been evolutionarily conserved, suggesting that caffeine may extend lifespan in other eukaryotes, including man.

Reference

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Caffeine extends yeast lifespan by targeting TORC1

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Summary
Dietary nutrient limitation (dietary restriction) is known to increase lifespan in a variety of organisms. Although the molecular events that couple dietary restriction to increased lifespan are not clear, studies of the model eukaryote Saccharomyces cerevisiae have implicated several nutrient-sensitive kinases, including the target of rapamycin complex 1 (TORC1), Sch9, protein kinase A (PKA) and Rim15. We have recently demonstrated that TORC1 activates Sch9 by direct phosphorylation. We now show that Sch9 inhibits Rim15 also by direct phosphorylation. Treatment of yeast cells with the specific TORC1 inhibitor rapamycin or caffeine releases Rim15 from TORC1-Sch9-mediated inhibition and consequently increases lifespan. This kinase cascade appears to have been evolutionarily conserved, suggesting that caffeine may extend lifespan in other eukaryotes, including man.

Introduction
Reduction of food intake, commonly referred to as dietary restriction (DR), has been shown to slow ageing and extend lifespan in virtually every biological system examined (Masoro, 2005). However, the underlying mechanisms that couple DR to lifespan extension remain poorly defined. Recently, the relatively simple eukaryote Saccharomyces cerevisiae (bakers’ yeast) has emerged as a powerful model system to study the genetic and physiolog-
cal factors that alter lifespan. Studies in yeast have demonstrated that genetic impairment of conserved nutrient-responsive signal transduction pathways can phenocopy DR and extend both chronological lifespan (CLS; viability in stationary phase) and replicative lifespan (RLS; number of daughters/buds produced). Specifically, reducing the kinase activities of the target of rapamycin complex 1 (TORC1), the TORC1 substrate Sch9 or protein kinase A (PKA) have been found to extend CLS (Fabrizio et al., 2001; Longo and Finch, 2003; Kaeberlein et al., 2005; Powers et al., 2006; Urban et al., 2007). In contrast, reducing the kinase activity of Rim15 decreases CLS (Reinders et al., 1998; Fabrizio et al., 2001; Wei et al., 2008). Importantly, RLS is not further extended by DR in TORC1 or Sch9 mutants, strongly suggesting that DR extends RLS via TORC1-Sch9 (Kaeberlein et al., 2005). TORC1-Sch9 and PKA are thought to signal in parallel pathways to positively regulate glycolysis, ribosome biogenesis and growth (Jorgensen et al., 2004). Additionally, TORC1-Sch9 and PKA signals converge at Rim15 to inhibit stress responses, G0 programmes, CLS and, as recently reported, also autophagy (Reinders et al., 1998; Pedruzzi et al., 2003; Wanke et al., 2005; Yorimitsu et al., 2007). Notably, PKA inhibits the kinase activity of Rim15 by direct phosphorylation (Reinders et al., 1998), while TORC1 contributes to the cytoplasmic sequestration of Rim15 via partially characterized mechanism(s) (Wanke et al., 2005). Rim15 appears to be conserved among eukaryotes as it shares homology with the mammalian serine/threonine kinase large tumour suppressor (LATS) (Pedruzzi et al., 2003; Cameroni et al., 2004; ORC1, Sch9 and PKA have clear orthologues in mammals – mammalian TORC1 (mTORC1), S6K and PKA respectively (Powers, 2007).

Yeast and mammalian TOR (mTOR) belong to a family of related kinases known as phosphotyrosinositol kinase-related kinases (PIKKs). In mammals, this family also includes DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases. The catalytic activity of these PIKKs can be inhibited to varying degrees by a number of pharmacological agents, including the xanthine alkaloid caffeine. Curiously, although caffeine inhibits multiple PIKKs in vitro (Sarkaria et al., 1999; Block et al., 2004), it appears to preferentially inhibit mTOR over other PIKKs in vivo (Cortez, 2003; Kaufmann et al., 2003). In contrast, the macrocyclic lactone rapamycin is a potent and specific inhibitor of TORC1/mTORC1 (Wullschleger...
et al., 2006). Clinically, rapamycin is used as an immunosuppressant and is presently being evaluated as an anti-tumour agent (Guerin and Sabatini, 2007). Of relevance to this study is the finding that low concentrations of rapamycin significantly extend CLS in yeast (Powers et al., 2006).

Caffeine has been proposed to target many cellular activities with cAMP phosphodiesterase being perhaps the most famous target (Bode and Dong, 2007). However, the notion that caffeine inhibits cAMP phosphodiesterase is controversial. Indeed, recent studies in yeast (Kuranda et al., 2006; Reinke et al., 2006) have demonstrated that TORC1, and not cAMP phosphodiesterase, is a major target of caffeine. Using both genetic and biochemical approaches to build on these recent results, we confirm that TORC1, and not TORC2, is the growth-limiting target of caffeine in yeast. Consistently, like low doses of rapamycin, low doses of caffeine significantly extended CLS. Characterization of the pathways downstream of TORC1 revealed that partial loss of TORC1 activity increases CLS via a previously undescribed TORC1–Sch9–Rim15 kinase cascade. This cascade is structurally conserved and this may explain recent epidemiological studies, which correlated moderate coffee (caffeine) consumption with decreased relative risk of mortality in humans (Fortes et al., 2000; Paganini-Hill et al., 2007).

Results and discussion

Caffeine inhibits TORC1

To extend the observations that caffeine preferentially inhibits (m)TOR over other PIKKs in vivo, we asked whether caffeine inhibits TORC1 and/or structurally and functionally distinct TORC2 in yeast (De Virgilio and Loewith, 2006). Like rapamycin, caffeine caused rapid, dose-dependent dephosphorylation of the C-terminal phosphorylation sites in Sch9, whereas partial dephosphorylation of the TORC2 substrates Ypk1/2 was observed at only the highest doses tested (Fig. 1A and B) (Urban et al., 2007). This demonstrates that in vivo, TORC1 is more sensitive to caffeine than TORC2. To determine whether TORC1 is a primary target of caffeine in yeast, we took

Fig. 1. Caffeine inhibits TORC1.

A. As indicated, yeast cultures were treated for 15 min with drug vehicle or varying concentrations of rapamycin or caffeine. Western blots detecting the extent of Sch9 phosphorylation were used to quantify TORC1 activity in vivo.

B. Similar to A, western blots using antiserum that recognizes Sch9 and Ypk1/Ypk2 when phosphorylated at the TORC1 and TORC2 sites respectively were used to quantify TORC1 and TORC2 activities following rapamycin or caffeine treatment (* denotes signal from an unknown protein that cross-reacts with the antiserum).

C. Yeast cells can be genetically engineered to bypass the essential functions of TORC1 and/or TORC2. Spotting 10-fold dilutions of these cells onto YPD plates containing drug vehicle, 200 nM rapamycin or 20 mM caffeine indicates that unlike TORC1 bypass (TB105-3b+pJU948+YCplac33+pRS414), TORC2 bypass [RL276-2d+YEp352(YPK2D239A-HA)] confers no resistance to either of these compounds.

D and E. In vitro TORC1 kinase assays using Sch9 as substrate were used to determine the IC50 of caffeine (D) and rapamycin (E). All assay points in (D) and (E) were done in triplicate and expressed as mean ± SD.
advantage of our ability to genetically bypass the essential function of TORC1 in vivo (see Experimental procedures) (Urban et al., 2007). Bypass of TORC1, but not bypass of TORC2, renders cells resistant to high doses of rapamycin and caffeine (Fig. 1C). Consistent with these in vivo data and in very good agreement with previous reports (Sarkaria et al., 1999; Reinke et al., 2006), we also observed that caffeine inhibited TORC1 activity towards its physiological substrate Sch9 in vitro with an apparent IC50 of 0.22 mM (Fig. 1D; IC50 for rapamycin = 5.2 nM; Fig. 1E).

We infer from these results that TORC1 is the major growth-limiting target of caffeine in yeast.

The TORC1 target Sch9 directly inhibits Rim15 function

As both TORC1 inhibition (by rapamycin or caffeine) and loss of Sch9 induce Rim15-dependent gene expression (Fig. 2A and B; Pedruzzi et al., 2003; Wanke et al., 2005), we investigated if TORC1 might inhibit Rim15 function via Sch9. We found that Sch9 physically interacted with Rim15 in co-immunoprecipitation (co-IP) experiments (Fig. 3A). Moreover, Sch9, and even more efficiently Sch9DE and Sch9S1061A (versions of Sch9 in which residues phosphorylated by TORC1 have been substituted with acidic amino acids; Urban et al., 2007), but not kinase-inactive Sch9KD, phosphorylated Rim15 in vitro within a loop (Rim15L) that is inserted between kinase subdomains VII and VIII (Fig. 3B). This kinase insert is typical of proteins of the LATS kinase family (Tamaskovic et al., 2003; Cameroni et al., 2004). Mass spectroscopy combined with specific Ser to Ala mutation analysis identified Ser1061 as the main residue phosphorylated in vitro by Sch9 (Fig. 3C). To determine whether this amino acid residue is also a target of Sch9 within cells, we raised an antiserum specific to this phosphorylated sequence (Fig. 3D and E). Using this specific anti-pSer1061 antiserum, we found that phosphorylation of Ser1061 in Rim15 in vivo depends largely on the presence of Sch9 (Fig. 3F), and is highly sensitive to rapamycin and caffeine treatment (Fig. 3G), as well as to glucose limitation (Fig. 3H). Importantly, dephosphorylation of Ser1061 in Rim15 induced by rapamycin or caffeine was not observed in cells expressing the TORC1-independent Sch9DD3E (Fig. 3G). Thus, TORC1 regulates the phosphorylation of Ser1061 in Rim15 via Sch9.

Next, we wished to determine if phosphorylation of Ser1061 is physiologically important for Rim15 regulation. Mutation of Ser1061 to Ala significantly and constitutively impaired cytoplasmic retention of Rim15 (Fig. 4A and B), which per se was insufficient to activate Rim15-dependent readouts in exponentially growing cells (as determined by SSA3 expression and glycogen staining; Fig. 4C and data not shown). Rapamycin or caffeine treatment caused both nuclear translocation and activation of Rim15; and expression of Sch9DD3E significantly blocked these effects in wild-type, but not in Rim15S1061A-expressing cells (Fig. 4A–C). Together, these data show that Ser1061 in Rim15 is a physiologically relevant Sch9 target, and indicate that induction of the Rim15-dependent programme requires downregulation of Sch9 (to allow accumulation of Rim15 in the nucleus) as well as alteration of at least one additional Sch9-independent, yet TORC1-controlled mechanism (to allow activation of the Rim15-dependent G0 programme).

How does Ser1061 phosphorylation regulate the subcellular localization of Rim15? We previously reported that the phosphorylation status of Thr1075 contributes to Rim15 cytoplasmic anchorage by 14-3-3 proteins (Wanke et al., 2005). Thr1075 phosphorylation is independently regulated by the cyclin-cyclin-dependent kinase Pho80-Pho85 (by direct phosphorylation) and by TORC1 (not through Pho80-Pho85, but presumably via inhibition of a protein phosphatase) (Wanke et al., 2005). Given the proximity between the Thr1075 residue and the newly identified Sch9 target residue Ser1061, Rim15 likely engages in binding the two monomeric subunits within a single 14-3-3 protein dimer (as is typically the case for other proteins). Accordingly, phosphorylation of Ser1061 and Thr1075 in Rim15 may cooperatively mediate tandem 14-3-3 binding to guaran-
In line with this model, individual Ser1061 or Thr1075 to Ala mutations in Rim15 significantly and constitutively impaired cytoplasmic retention of Rim15 (Fig. 4A; Wanke et al., 2005). Moreover, as expected, if TORC1 targets Ser1061 and Thr1075 by different mechanisms, TORC1 inhibition (using caffeine or rapamycin) exacerbated the cytoplasmic retention defects of the Ala variants of both Rim15-Ser1061 and Rim15-Thr1075 (Fig. 4A and data not shown; Wanke et al., 2005).

Caffeine extends yeast lifespan via a TORC1–Sch9–Rim15 kinase cascade

Rim15 orchestrates various physiological processes, including antioxidant defence mechanisms, accumulation of storage carbohydrates (such as glycogen) and upregulation of stress-responsive gene expression, all of which have been shown to critically affect CLS (Reinders et al., 1998; Fabrizio and Longo, 2003; Pedruzzi et al., 2003; Cameroni et al., 2004; Powers et al., 2006). This suggests

Fig. 3. Sch9 targets Rim15 both in vitro and in vivo.
A. Sch9 and Rim15 physically interact. Sch9-HA2 (lanes 1 and 3) and Mpk1-HA2 (lane 2; negative control) were immuno-precipitated from cells coexpressing Rim15-myc13 (lanes 1 and 2) or Ego1-myc13 (lane 3; negative control). Cell lysates (input) and immunoprecipitates (IP) were subjected to SDS-PAGE and immunoblots were probed using anti-HA or anti-myc antibodies (* denotes detection of the heavy chain of the immunoprecipitation antibody).
B. Sch9, Sch9ΔS and Sch9ΔD3E, but not inactive Sch9KD, phosphorylate a bacterially expressed, GST-Rim15 kinase insert domain (GST-Rim15KI) in vitro.
C. Sch9 targets Ser1061 in Rim15. Substitution of Ser1061 with Ala abolishes phosphorylation of GST-Rim15KI-S1061A by Sch9ΔD3E (sKI harbours amino acids 1049–1078 of the original Rim15 sequence).
D and E. Phospho-specific antibodies directed towards Ser1061 in Rim15 recognize GST-Rim15 purified from exponentially growing yeast prior to, but not following, phosphatase treatment (D), and bacterially expressed GST-Rim15KI following, but not prior to, in vitro phosphorylation by Sch9 (and/or Sch9ΔS/Sch9ΔD3E; E). PPI denotes phosphatase inhibitor.
F–H. In vivo phosphorylation of Ser1061 in Rim15 requires the presence of Sch9 (F) and is sensitive to rapamycin (200 nM) or caffeine (20 mM) treatment (G), and glucose limitation (H).
that TORC1-Sch9 may negatively regulate CLS mainly by activating Sch9 and consequently inhibiting Rim15 function. In support of this assumption, expression of Sch9<sup>2D3E</sup>, similar to loss of Rim15, reduced CLS, while expression of Rim15<sup>S1061A</sup> extended CLS in both wild-type and Sch9<sup>2D3E</sup> expressing cells (Fig. 5A). Finally, inhibition of TORC1 by low doses of caffeine (0.2–0.4 mM) or rapamycin (0.55 nM) significantly extended CLS in wild-type [i.e. the median survival of wild-type cells was increased on average by 0.86 (±0.26 SEM; n = 11) or 1.71 (±0.36 SEM; n = 4) days respectively] but not in rim15<sup>D</sup> cells (Fig. 5B). At these concentrations of caffeine and rapamycin, TORC1 activity is reduced by approximately 3% (as interpolated from the results presented in Fig. 1A). Based on these data, we propose that extension of lifespan following TORC1 downregulation either physiologically (i.e. DR) or pharmacologically (e.g. using caffeine or rapamycin) is mediated by this newly identified Sch9-Rim15 effector branch.

**Can caffeine extend lifespan in humans?**

TORC1, Sch9 and Rim15 are conserved in higher eukaryotes – mTORC1, S6K and LATS kinases respectively in humans (Cameroni <em>et al.</em>, 2004; Wullschleger <em>et al.</em>, 2006; Urban <em>et al.</em>, 2007); and S6K is a well-documented substrate of mTORC1 (Wullschleger <em>et al.</em>, 2006). Thus, it is possible that an analogous mTORC1/S6K/LATS kinase cascade may also influence longevity in metazoans. Indeed, several studies have already demonstrated that decreased TOR or S6K activity increases lifespan in worms and flies (Vellai <em>et al.</em>, 2003; Jia <em>et al.</em>, 2004; Kapahi <em>et al.</em>, 2004). This begs the question: can caffeine extend lifespan in humans? Caffeine is the most widely used psychoactive drug worldwide with coffee being the main source of caffeine in the Western diet. Tantalizingly, epidemiological studies have correlated habitual coffee consumption with a decreased relative risk of mortality (Fortes <em>et al.</em>, 2000; Paganini-Hill <em>et al.</em>, 2007). Drinking one cup of coffee results in an approximate peak plasma concentration of 1–10 μM caffeine in humans (with an
estimated half-life of 2.5–4.5 h) (Arnaud, 1987; Fredholm et al., 1999). Assuming that caffeine inhibition of mTORC1 in vivo is comparable to its inhibition of yeast TORC1 in vitro (Fig. 1D), moderate coffee consumption is expected to cause a 4–8% inhibition of mTORC1 activity. This range of inhibition compares well with the extent of inhibition that we calculate to be necessary for lifespan extension in yeast (~3%), and thus provides mechanistic support for the correlative links between coffee consumption and longevity described above. At this concentration of caffeine, inhibition of other PIKK family members (ATM, ATR, DNA-PKcs) does not appear to have deleterious consequences. Finally, caffeine has recently been shown to suppress cell transformation (Nomura et al., 2005), suggesting that, like rapamycin (Guertin and Sabatini, 2007), caffeine may also be a (well-tolerated) and effective anti-cancer agent.

**Experimental procedures**

**Cloning and yeast experiments**

Yeast strains and plasmids used in this study are listed in Tables 1 and 2. Strains were grown at 30°C in standard rich medium with 2% glucose (YPD) or synthetic medium with 2% glucose (SD), 4% galactose (SGal) or 2% raffinose (SRaf) as carbon source. Standard yeast genetic manipulations were used. For site-directed mutagenesis, the QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used with the

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**Table 1. Strains used in this study.**

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<th>Genotype</th>
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Plasmids used in this study.

Table 2. Plasmids used in this study.

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appropriate primers that introduced the mutations. The presence of mutagenized sites was confirmed by sequencing.

Growth assay

TORC1-bypass strains: wild type (RL276-2d + YCplac33), TORC2-bypass [RL276-2d + YEp352(YPK2<sup>2023A,HA</sup>)], TORC1-bypass (TB105-3b + pJU948 + YCplac33 + pRS414) and TORC1/2-bypass [TB105-3b + pJU948 + YEp352 (YPK2<sup>2023A,HA</sup>) + pRS414] were grown to mid-log phase and diluted to 0.25 OD<sub>600</sub> in medium. Serial dilutions (1:1, 10, 100) were spotted on YPD plates containing rapamycin or caffeine. Plates were incubated 2–3 days at 30°C.

Sch9 and Ypk2 carboxy-terminal phosphorylation

To analyse Sch9-5HA C-terminal phosphorylation, TB50 cells containing plasmids pJU450 and pJU676 were grown in SC-Ura, -His, -Leu to mid-log phase, harvested and re-suspended in YPAD + 0.2% Gln at 0.5 OD<sub>600</sub>. Cells were grown for 60 min at 30°C prior to addition of medium containing rapamycin or caffeine and subsequent incubation for another 30 min. Chemical fragmentation analysis was done as described (Urban et al., 2007). To analyse Ypk2 phosphorylation, MP8 cells were grown in YPD + 0.2% glucose at 30°C to an OD<sub>600</sub> between 0.6 and 0.8, at which point rapamycin or caffeine was added to the indicated final concentration. Cells were shaken for an additional 30 min and then harvested as described in Urban et al. (2007), but without 2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-HA antibody or rabbit anti-phospho-T659 Ypk2 antisierum (this antisierum detects both Sch9 phosphorylated at T737 by TORC1 as well as Ypk2 phosphorylated at T659 by TORC2; R. Loewith, unpublished).

TORC1 kinase assay

The TORC1 was purified from RL194-4c cells (grown to an OD<sub>600</sub> of 1.5–2.0 in YPD, 150 ml per assay point) using a protocol very similar to that described (Urban et al., 2007). To cleared protein extracts were added 25 µl of prepared paramagnetic beads (Dynabeads M-270 Epoxy, 2 x 10<sup>8</sup> ml<sup>-1</sup>), coated with rabbit IgG; Sigma) and tubes were subsequently rotated for 2 h at 4°C. Beads were collected by using a magnet, washed extensively with cold lysis buffer without inhibitors, aliquotted to 1.5 ml tubes and frozen at –80°C. Kinase reactions were performed in a final volume of 30 µl containing TORC1-coupled beads, 600 ng Sch9 (Urban et al., 2007), 25 mM Heps/KOH pH 7.2, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5% Tween20, 1x Roche protease inhibitor-EDETA, 100 µM ATP, 2 µl of [γ<sup>32</sup>P]-ATP and inhibitors at various concentrations. In rapamycin experiments, each reaction contained 200 ng of GST-FKBP12 and 1.1% DMSO. Caffeine was dissolved in H<sub>2</sub>O and used at the indicated concentrations. All assay points were done in triplicate. Assays were started with addition of ATP, maintained at 30°C for 15 min and terminated by the addition of 8 µl of 5x SDS-PAGE buffer. Samples were heated to 95°C for 5 min; proteins were resolved in SDS-PAGE, stained with Coomassie Blue and analysed using a Bio-Rad Molecular Imager. IC<sub>50</sub> values were calculated by using the GraphPad Prism 5.0 program.

Immunoprecipitation and immunoblot analyses

For co-IP experiments between Rim15 and Sch9, strain KT1960 was co-transformed with pVW904 (expressing
GST pull-down and phospho-specific antibodies

Full-length Rim15 was purified from strain KT1960, which expresses (from plasmid pNB566) GST-Rim15 under the GAL1 promoter. Induction of GAL1-driven expression and cell lysis were essentially performed as described (Wanke et al., 2005). GST-tagged Rim15 was purified from clarified extracts using glutathione sepharose 4B beads (Amersham Biosciences). Dephosphorylation of GST-Rim15 (bound to sepharose 4B beads) was carried out by 30 min incubation at 30°C with 1 U of λ-phosphatase (Biolabs, NewEngland). In control reactions, phosphatase inhibitors (10 mM NaF, 10 mM Na-orthovanadate, 10 mM p-NO2-phenylphosphate, 10 mM glycerophosphate and 10 mM Na-pyrophosphate) were added. Antibodies against Rim15 phosphorylated on Ser1061 were raised against a phosphorlated synthetic peptide (A-S-L-R-R-S-E-pS-Q-L-S-F; where pS represents phospho-Ser1061 of Rim15), adsorbed with the unphosphorylated form of the peptide, and affinity-purified with the phosphorylated peptide by Eurogentec.

Sch9 protein kinase assays and quantification of substrate phosphorylation

To assay in vitro phosphorylation of Rim15 by Sch9, TB50 cells containing plasmid-based alleles of SCH9-3HA were grown and treated essentially as described (Urban et al., 2007). Sch9 proteins were purified as described (Urban et al., 2007). Kinase assays were performed with Sch9-3HA-bound beads at 30°C for 30 min in kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 1 mM ATP and 10 μCi ATP) and GST-Rim15-derived substrates (purified from Escherichia coli). Reactions were stopped by adding SDS gel-loading buffer and boiling for 5 min and then subjected to SDS-PAGE. Substrate phosphorylation levels were quantified using a PhosphorImager (Cyclone Phosphor System; PerkinElmer) and analysed with OptiQuant Image Analysis software [Packard]. Digital images of immunoblots were acquired with a CanoScan LiDE scanner (Canon) and Photoshop 7.0 (Adobe) and densitometric analysis of protein bands was done with OptiQuant Image Analysis software.

Ageing assays

To analyse CLS, strain YFL033C was rendered prototrophic and co-transformed with plasmid-based alleles of RIM15 and SCH9. Accordingly, strains are: wild type (YFL033C + pVW1388 + pRS413 + pRS416); rim15Δ (YFL033C + pRS415 + pRS416 + pRS413; or strain RL287-2A + YeP195 in Fig. 5B); SCH92D3E (YFL033C + pVW1388 + pJU841 + pRS413); RIM15S1061A (YFL033C + pVW1389 + pRS416 + pRS413); and SCH92D3E/RIM15S1061A (YFL033C + pVW1389 + pJU841 + pRS413) (see Table 1 for further details). Cells were grown at 30°C in SD medium. Overnight cultures were diluted to early exponential phase (0.2 OD600), and rapamycin or caffeine (or drug vehicle alone) was added during the exponential growth phase. Each experiment was performed at least in triplicate. Cell cultures were incubated at 30°C without replacing the growth medium throughout the experiment. Culture aliquots were collected regularly and serial dilutions were plated on YPD. Colony-forming units (cfu ml⁻¹) were expressed as percentage of the values at day 4 (early stationary phase).

Miscellaneous

For glycogen assays and β-galactosidase assays, strain RL267-3d was co-transformed with plasmid-based alleles of RIM15 and SCH9. Accordingly, strains are: wild type (RL267-3d + pVW1388 + pJU675); rim15Δ (RL267-3d + pRS315 + pJU675); SCH92D3E (RL267-3d + pVW1388 + pJU841); RIM15S1061A (RL267-3d + pVW1389 + pJU675); and SCH92D3E/RIM15S1061A (RL267-3d + pVW1389 + pJU841) (see Table 1 for details). Cells were grown in SD medium to exponential phase and then treated with 100 ng ml⁻¹ rapamycin or 10 mM caffeine or drug vehicle for 15 h at 30°C. Ten OD600 equivalents of cells were harvested by filtration onto Millipore HA filters (Bedford, MA), placed upon a solid agar matrix and exposed to iodine vapour for 2 min (Lirie and Pringle, 1980). β-Galactosidase assays were performed as described earlier (Reinders et al., 1998). Northern analyses and immunofluorescence were performed as described (Dubouloz et al., 2005). DNA was stained with 4,6-diamidino-2-phenylindole, which was added to the cultures (4 h prior to fluorescence microscopy) (Wanke et al., 2005) at a concentration of 1 μg ml⁻¹.

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