Article

Caffeine extends yeast lifespan by targeting TORC1

WANKE, Valeria, *et al*.

Abstract

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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 physiological 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, G_0 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); TORC1, 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 phosphytidylinositol 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

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et al., 2006). Clinically, rapamycin is used as an immunosuppressant and is presently being evaluated as an antitumour agent (Guertin 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 the 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

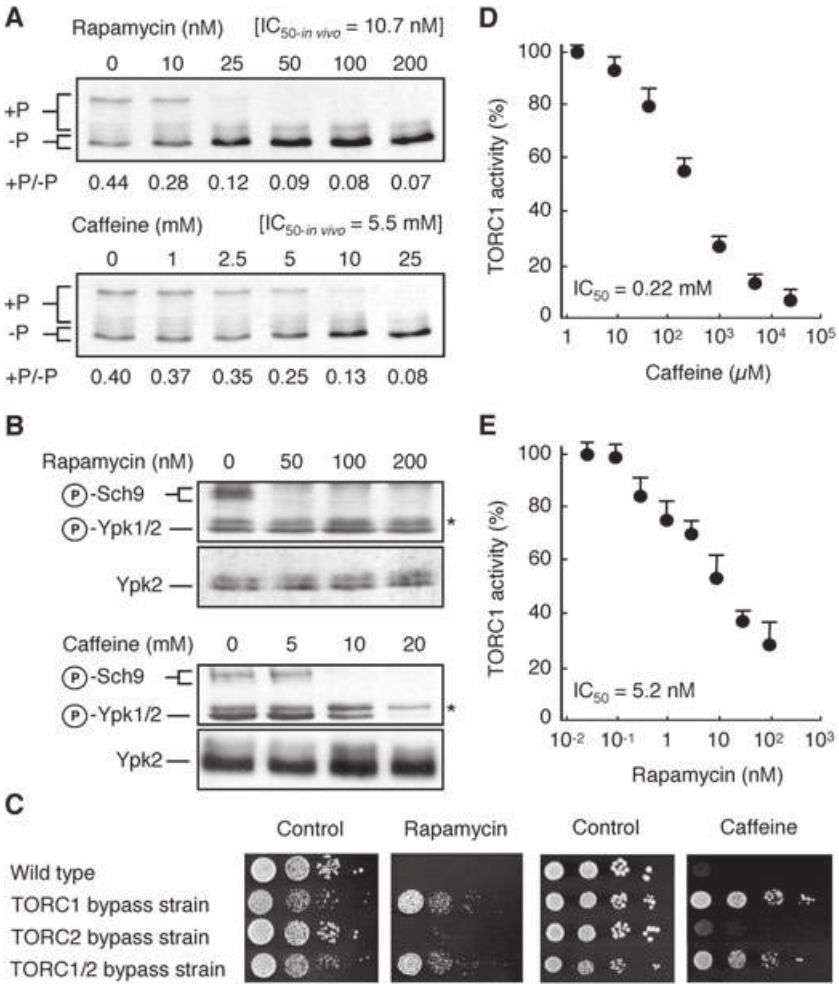

Fig. 1. Caffeine inhibits TORC1.
A. As indicated, yeast cultures were treated for 15 min with drug vehicle or varying concentrations of rapamycin or caffeine. Western blots detecting the extent of Sch9 phosphorylation were used to quantify TORC1 activity *in vivo*.
B. Similar to A, western blots using antiserum that recognizes Sch9 and Ypk1/Ypk2 when phosphorylated at the TORC1 and TORC2 sites respectively were used to quantify TORC1 and TORC2 activities *in vivo* 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(YPK2^{D239A}-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 IC_{50} of caffeine (D) and rapamycin (E). All assay points in (D) and (E) were done in triplicate and expressed as mean + SD.

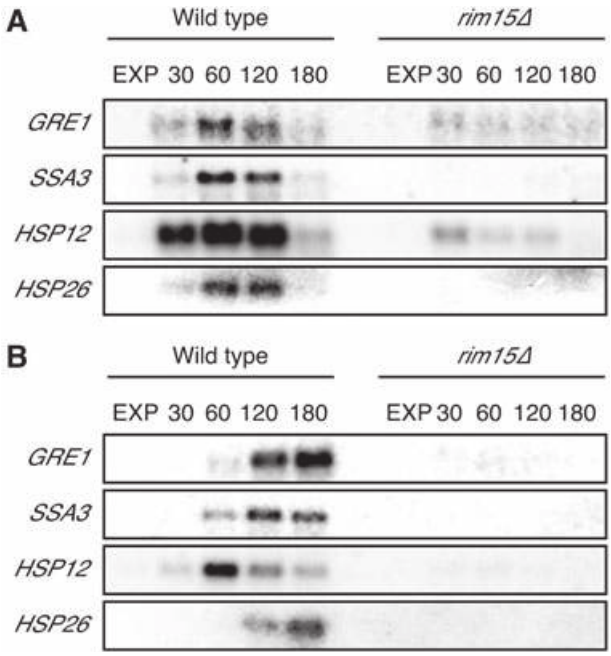

Fig. 2. Rim15 is required for induction of *GRE1, SSA3, HSP12* and *HSP26* following TORC1 inactivation by rapamycin (A) or caffeine (B).
A and B. RNA was collected from exponentially growing (OD_{600} < 0.8) wild-type (TS120-2d + pJU450 + pJU675) and isogenic *rim15*Δ (RL267-10d + pJU450 + pRS416) mutant cells following treatment with rapamycin (0.2 μg ml^{-1}) or caffeine (20 mM) for the times indicated. Equal amounts of RNAs (10 μg) were probed and the corresponding Northern analyses of indicated messages are shown.

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 IC_{50} of 0.22 mM (Fig. 1D; IC_{50} 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 $Sch9^{3E}$ and $Sch9^{2D3E}$ (versions of Sch9 in which residues phosphorylated by TORC1 have been substituted with acidic amino acids; Urban *et al*., 2007), but not kinase-inactive $Sch9^{KD}$, phosphorylated Rim15 *in vitro* within a loop ($Rim15^{KI}$) 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 Ser^{1061} 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-$pSer^{1061}$ antiserum, we found that phosphorylation of Ser^{1061} 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 Ser^{1061} in Rim15 induced by rapamycin or caffeine was not observed in cells expressing the TORC1-independent $Sch9^{2D3E}$ (Fig. 3G). Thus, TORC1 regulates the phosphorylation of Ser^{1061} in Rim15 via Sch9.

Next, we wished to determine if phosphorylation of Ser^{1061} is physiologically important for Rim15 regulation. Mutation of Ser^{1061} 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 $Sch9^{2D3E}$ significantly blocked these effects in wild-type, but not in $Rim15^{S1061A}$-expressing cells (Fig. 4A–C). Together, these data show that Ser^{1061} 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 G_0 programme).

How does Ser^{1061} phosphorylation regulate the subcellular localization of Rim15? We previously reported that the phosphorylation status of Thr^{1075} contributes to Rim15 cytoplasmic anchorage by 14-3-3 proteins (Wanke *et al*., 2005). Thr^{1075} 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 Thr^{1075} residue and the newly identified Sch9 target residue Ser^{1061}, 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 Ser^{1061} and Thr^{1075} in Rim15 may cooperatively mediate tandem 14-3-3 binding to guaran-

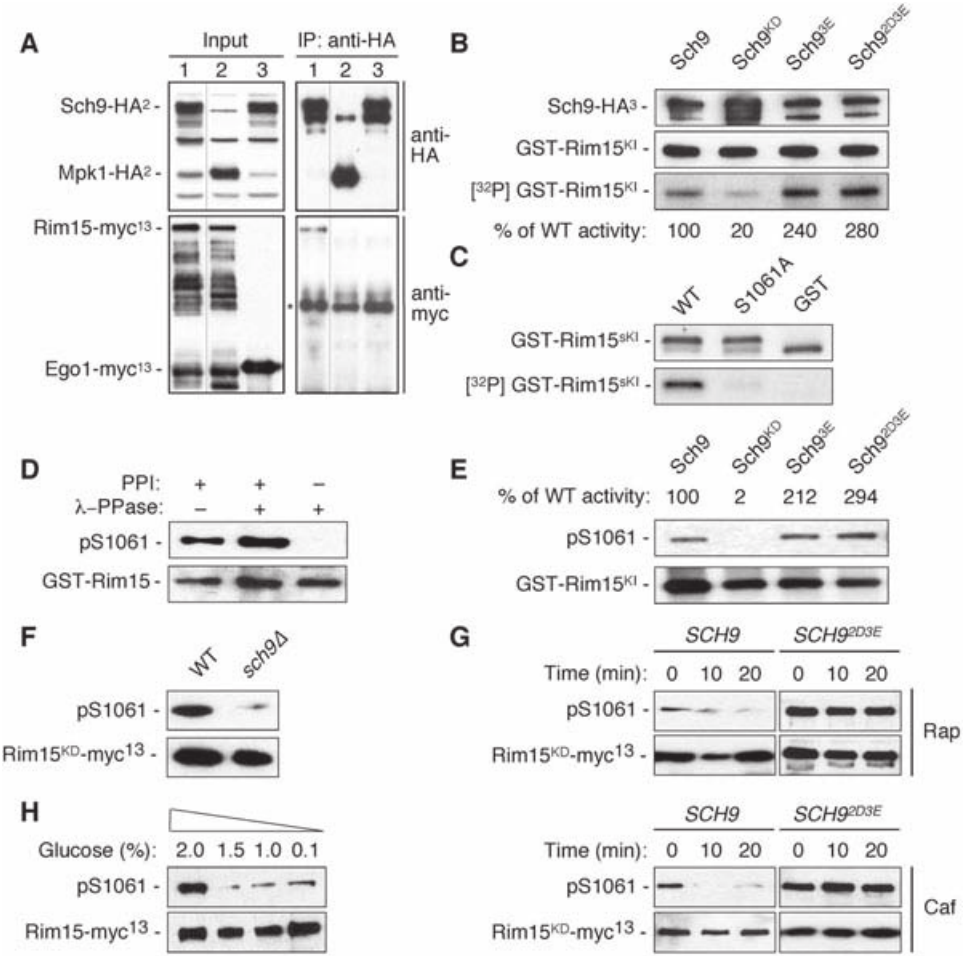

Fig. 3. Sch9 targets Rim15 both *in vitro* and *in vivo*.
A. Sch9 and Rim15 physically interact. Sch9-HA^{2} (lanes 1 and 3) and Mpk1-HA^{2} (lane 2; negative control) were immuno-precipitated from cells coexpressing Rim15-myc^{13} (lanes 1 and 2) or Ego1-myc^{13} (lane 3; negative control). Cell lysates (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, Sch9^{3E} and Sch9^{2D3E}, but not inactive Sch9^{KD}, phosphorylate a bacterially expressed, GST-Rim15 kinase insert domain (GST-Rim15^{KI}) *in vitro*.
C. Sch9 targets Ser^{1061} in Rim15. Substitution of Ser^{1061} with Ala abolishes phosphorylation of GST-Rim15$^{sKI\text{-}S1061A}$ by Sch9^{2D3E} (sKI harbours amino acids 1049–1078 of the original Rim15 sequence).
D and E. Phospho-specific antibodies directed towards Ser^{1061} in Rim15 recognize GST-Rim15 purified from exponentially growing yeast prior to, but not following, phosphatase treatment (D), and bacterially expressed GST-Rim15^{KI} following, but not prior to, *in vitro* phosphorylation by Sch9 (and/or Sch9^{3E}/Sch9^{2D3E}; E). PPI denotes phosphatase inhibitor.
F–H. *In vivo* phosphorylation of Ser^{1061} 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).

tee optimal sequestration of Rim15 in the cytoplasm. In line with this model, individual Ser^{1061} or Thr^{1075} 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 Ser^{1061} and Thr^{1075} by different mechanisms, TORC1 inhibition (using caffeine or rapamycin) exacerbated the cytoplasmic retention defects of the Ala variants of both Rim15-Ser^{1061} and Rim15-Thr^{1075} (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

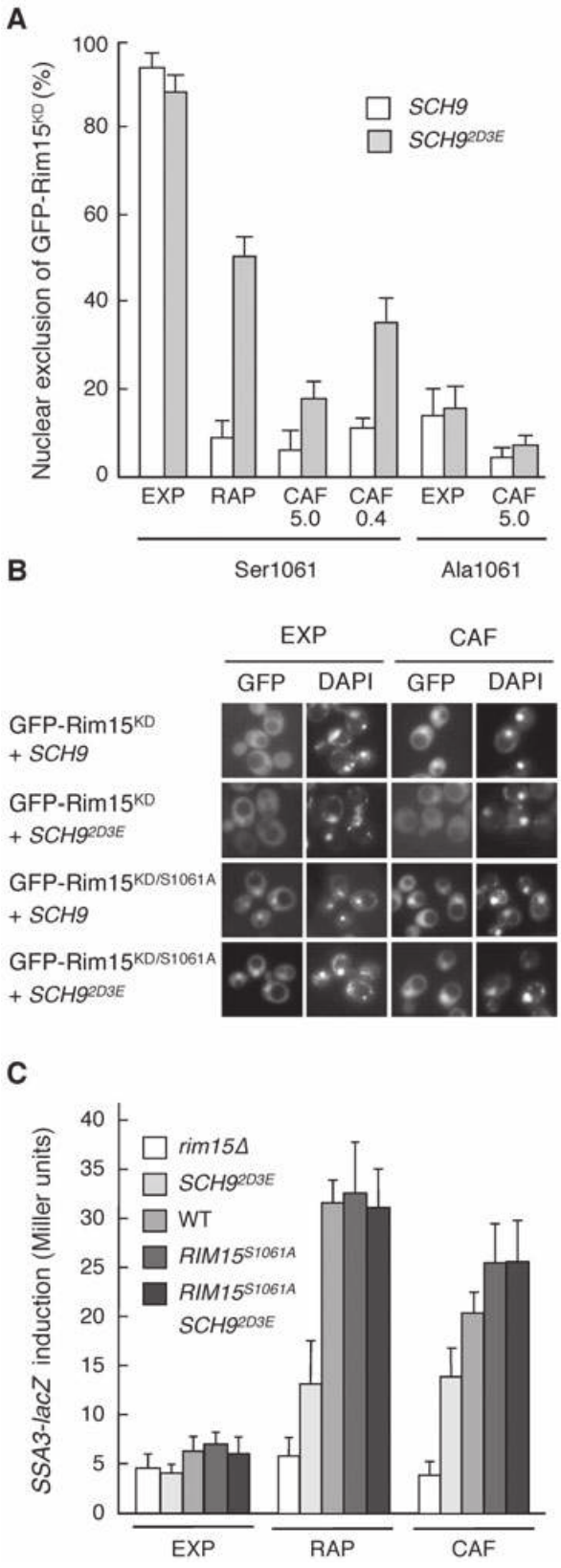

Fig. 4. The TORC1-Sch9 effector branch antagonizes the G_0 programme by promoting nuclear exclusion of Rim15.
A. Exponentially growing *rim15*Δ cells expressing kinase inactive GFP-Rim15^{KD} or GFP-Rim15$^{KD/S1061A}$ and either wild-type Sch9, or Sch9^{2D3E}, were treated for 30 min with rapamycin (200 nM; RAP) or the indicated concentrations of caffeine (in mM; CAF) and subsequently visualized by fluorescence microscopy.
B. Exponentially growing *rim15*Δ cells expressing GFP-Rim15^{KD} or GFP-Rim15$^{KD/S1061A}$ and either wild-type Sch9 or Sch9^{2D3E}, were treated for 30 min with caffeine (5 mM; CAF) and subsequently visualized by fluorescence microscopy.
C. Induction of *SSA3-lacZ* following treatment of cells for 15 h with rapamycin (100 nM; RAP) or caffeine (10 mM; CAF). Relevant genotypes are indicated.

that TORC1-Sch9 may negatively regulate CLS mainly by activating Sch9 and consequently inhibiting Rim15 function. In support of this assumption, expression of Sch9^{2D3E}, similar to loss of Rim15, reduced CLS, while expression of Rim15^{S1061A} extended CLS in both wild-type and Sch9^{2D3E}-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*Δ 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 *et al.*, 2004; Wullschleger *et al.*, 2006; Urban *et al.*, 2007); and S6K is a well-documented substrate of mTORC1 (Wullschleger *et al.*, 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 *et al.*, 2003; Jia *et al.*, 2004; Kapahi *et al.*, 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 *et al.*, 2000; Paganini-Hill *et al.*, 2007). Drinking one cup of coffee results in an approximate peak plasma concentration of 1–10 μM caffeine in humans (with an

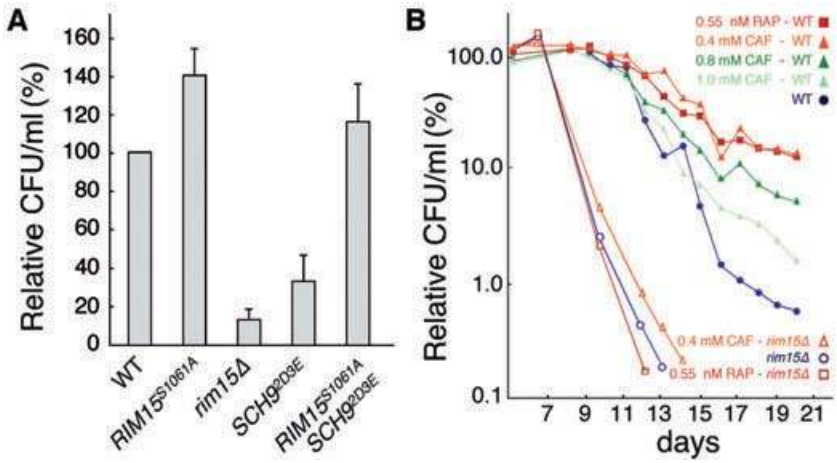

Fig. 5. Caffeine extends yeast lifespan by downregulating the TORC1–Sch9–Rim15 signalling cascade.
A. Loss of Rim15 or expression of $Sch9^{2D3}$ reduces, while expression of $Rim15^{S1061A}$ extends lifespan. Survival (i.e. cfu ml^{-1}) was assessed in 12-day-old cultures and expressed as relative values compared with wild-type cells.
B. Direct inhibition of TORC1 by low doses of caffeine and rapamycin extends chronological lifespan of *S. cerevisiae* wild type, but not of *rim15*Δ cells. Each data point represents the mean of three samples. Survival data (cfu ml^{-1}) are expressed as relative values compared with the values at day 4 (early stationary phase). Survival curves for 0.4 mM caffeine ($P = 0.0002$) and 0.55 nM rapamycin ($P = 0.0001$) were significantly different from the untreated control curves as assessed by the Wilcoxon matched pairs test (using the GraphPad Prism 5.0 program).

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

Table 1. Strains used in this study.

Strain	Genotype	Source	Figure
JK9-3da	MATa; *trp1, his4, ura3, leu2, rme1*	Beck and Hall (1999)	
IP11	MATa; *rim15Δ::kanMX2* [JK9-3da]	Pedruzzi *et al.* (2003)	3G, H
KT1960	MATα; *ura3, leu2, his3, trp1, rme1*	Pedruzzi *et al.* (2003)	
IP31	MATα; *rim15Δ::kanMX2* [KT1960]	Pedruzzi *et al.* (2003)	3A, D
TB50a	MATa; *trp1, his3, ura3, leu2, rme1*	Beck and Hall (1999)	3B, C, E
RL276-2d	MATa; *TRP1, HIS3, LEU2* [TB50]	This study	1C
TB105-3b	MATa; *gat1::HIS3MX gln3::kanMX* [TB50]	Beck and Hall (1999)	1C
MP8	MATa; *YPK2-6HA [HIS3MX]* [TB50]	This study	1B
TS120-2d	MATa; *sch9Δ::KanMX2* [TB50]	Urban *et al.* (2007)	2A, B
RL194-4c	MATa; *TCO89-TAP[KITRP1] 3HA-TOR1* [TB50]	This study	1D, E
FD19	MATa; *EGO1-myc*	Dubouloz *et al.* (2005)	3A
RL267-10d	MATa; *rim15Δ::kanMX2* [TB50]	This study	2A, B; 4A
RL267-3d	MATa; *his4 sch9Δ::kanMX6, rim15Δ::KanMX2* [TB50]	This study	3F; 4C
BY4741	MATa; *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Euroscarf	
YFL033C	MATa; *rim15Δ::kanMX4 MET15* [BY4741]	Euroscarf	5A, B
RL287-2A	MATa; *rim15Δ::kanMX4 MET15* [BY4741]	This study	5B

Table 2. Plasmids used in this study.

Plasmid	Vector; Insert	Source	Figure
pJU450	pRS415; *TRP1, HIS3*	Urban *et al.* (2007)	1A; 2A, B
pJU676	pRS416; *SCH9-5HA*	Urban *et al.* (2007)	1A
pJU948	pRS415; *SCH9-5HA (T723D, S726D, T737E, S758E, S765E)*	This study	1C
YEp352; YPK2$^{D239A-HA}$	YEp352; *YPK2^{D239A}-HA*	Kamada *et al.* (2005)	1C
pVW904	pYEplac181; *TDH3p-RIM15-myc13*	Wanke *et al.* (2005)	3A, H
pVW885	pCM189; *MPK1-myc13*	This study	3A
pVW881	pCM189; *SCH9-2HA*	This study	3A
pVW995	pGEX3X; *RIM15-KI*	Wanke *et al.* (2005)	3B, E
pTS130	YCplac33; *SCH9-3HA*	Urban *et al.* (2007)	3B, E
pRL119-1	YCplac33; *SCH9-3HA (K441A)*	Urban *et al.* (2007)	3B, E
pAH051	YCplac33; *SCH9-3HA (T723D, S726D, T737E, S758E, S765E)*	Urban *et al.* (2007)	3B, C, E
pAH048	YCplac33; *SCH9-3HA (T737E, S758E, S765E)*	This study	3B, E
pVW1313	pGEX3X; *RIM15-aa1049–1078*	This study	3C
pVW1327	pGEX3X; *RIM15-aa1049–1078 (S1061A)*	This study	3C
pNB566	YEplac195; *GAL1p-GST-RIM15*	Wanke *et al.* (2005)	3D
pVW909	YEplac181; *TDH3p-RIM15-myc (K823Y)*	This study	3F, G
pJU675	pRS416; *SCH9*	Urban *et al.* (2007)	3F, G; 4A–C
pJU841	pRS416; *SCH9 (T723D, S726D, T737E, S758E, S765E)*	Urban *et al.* (2007)	3G; 4A–C; 5A
pFD633	pNP305; *ADH1p-GFP-RIM15 (C1176Y)*	Pedruzzi *et al.* (2003)	4A, B
pVW1329	pNP305; *ADH1p-GFP-RIM15 (C1176Y, S1061A)*	This study	4A, B
pVW1388	pRS315; *RIM15*	This study	4C; 5A; 5B
pVW1389	pRS315; *RIM15 (S1061A)*	This study	4C; 5A

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

Growth assay

TORC-bypass strains: wild type (RL276-2d + YCplac33), TORC2-bypass [RL276-2d + YEp352(YPK2^{D239A}-HA)], TORC1-bypass (TB105-3b + pJU948 + YCplac33 + pRS414) and TORC1/2-bypass [TB105-3b + pJU948 + YEp352 (YPK2^{D239A}-HA) + pRS414] were grown to mid-log phase and diluted to 0.25 OD_{600} 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_{600}. 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% glutamine at 30°C to an OD_{600} 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 antiserum (this antiserum 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_{600} 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, 2×10^9 ml^{-1}, 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 Hepes/KOH pH 7.2, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.5% Tween20, 1× Roche protease inhibitor-EDTA, 100 µM ATP, 2 µCi [γ-^{32}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_2O 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 5× SDS-PAGE buffer. Samples were heated to 95°C for 5 min; proteins were resolved in SDS-PAGE, stained with Coomassie and analysed using a Bio-Rad Molecular Imager. IC_{50} 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

Rim15-myc[13] under control of the *TDH3* promoter) and either pVW881 or pVW885, which expresses Sch9-2HA or Mpk1-2HA respectively, under the control of the *tetO7* promoter. To induce expression of the *tetO7*-controlled genes, cells were grown for at least six generations in exponential growth phase ($OD_{600} < 1.0$) in the absence of doxycycline. Subsequently, cells were lysed essentially as described (Wanke *et al*., 2005) and HA-tagged proteins were purified from clarified extracts with the protein G-agarose IP kit (Roche Diagnostics GmbH) following the manufacturer instructions using monoclonal mouse anti-HA antibodies (HA.11; Covance). Bound proteins were eluted with sample buffer (5 min, 95°C) and subjected to standard immunoblot analysis for detection of co-precipitated Rim15-myc[13] using anti-myc antibodies (Myc-Tag 9B11; Cell Signaling). In parallel, strain FD19 (expressing a genomically myc[13]-tagged version of Ego1 and harbouring plasmids pVW881 or pVW885) was subjected to same treatment and served as a negative control.

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-NO_2-phenylphosphate, 10 mM glycerophosphate and 10 mM Na-pyrophosphate) were added. Antibodies against Rim15 phosphorylated on Ser^{1061} were raised against a phosphorylated synthetic peptide (A-S-L-R-R-S-E-pS-Q-L-S-F; where pS represents phospho-Ser^{1061} 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 $MgCl_2$, 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 prototroph and co-transformed with plasmid-based alleles of *RIM15* and *SCH9*. Accordingly, strains are: wild type (YFL033C + pVW1388 + pRS413 + pRS416); *rim15*Δ (YFL033C + pRS415 + pRS416 + pRS413; or strain RL287-2A + YEp195 in Fig. 5B); $SCH9^{2D3E}$ (YFL033C + pVW1388 + pJU841 + pRS413); $RIM15^{S1061A}$ (YFL033C + pVW1389 + pRS416 + pRS413); and $SCH9^{2D3E}/RIM15^{S1061A}$ (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 OD_{600}), 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}) are 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); *rim15*Δ (RL267-3d + pRS315 + pJU675); $SCH9^{2D3E}$ (RL267-3d + pVW1388 + pJU841); $RIM15^{S1061A}$ (RL267-3d + pVW1389 + pJU675); and $SCH9^{2D3E}/RIM15^{S1061A}$ (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 OD_{600} 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 (Lillie 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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