TOR complex 1 regulates the yeast plasma membrane proton pump and pH and potassium homeostasis

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ABSTRACT

We have identified in yeast a connection between two master regulators of cell growth: a biochemical one, the TORC1 protein kinase (activating protein synthesis, nutrient uptake and anabolism) and a biophysical one, the plasma membrane proton-pumping H^+-ATPase (Pma1, driving nutrient uptake and regulating pH homeostasis). Raising temperature to non-permissive values in a TOR thermosensitive mutant decreases Pma1 activity. Rapamycin, a TORC1 inhibitor, inhibits Pma1 dependent on its Fpr1 receptor and on protein phosphatase Sit4, a TORC1 effector. Mutation of either Sit4 or Tco89, a non-essential subunit of TORC1, decreases proton efflux, K^+ uptake, intracellular pH, cell growth and tolerance to weak organic acids. Tco89 does not affect Pma1 activity but activates K^+ transport.

Keywords: Rapamycin; H^+-ATPase; Sit4; intracellular pH; K^+ transport

Abreviations

TOR, Target Of Rapamycin (protein kinase); TORC1, TOR Complex 1; Pma1, plasma membrane H^+-ATPase of *Saccharomyces cerevisiae*;

Introduction

The first mechanisms controlling growth and proliferation of cells probably consisted on direct environmental effects, such as nutrient availability and physicochemical factors such as pH, temperature and water activity. In order to avoid deleterious effects of unfinished cellular activities, a control point named START was introduced at the G1 phase of the cell cycle to ensure that cell growth and division would only start under favorable conditions [1, 2]. Finally, with the development of higher organisms, a second control named "restriction point", based on hormonal compounds called "growth factors" was introduced [2-4]. Tumorigenic transformation of mammalian cells consists on unregulated growth and proliferation and the two control points, START and "restriction point", are dysregulated in virtually all human cancers [2]. Accordingly, oncogenes are mutated versions of regulatory genes encoding component of signal transduction pathways, including membrane receptors, protein kinases, G proteins and transcription factors [5]. The TOR protein kinase is an essential regulator of growth in all eukaryotes that is activated by nutrients at START of the cell cycle [6, 7]. It operates through two protein complexes: TORC1 activates energy metabolism, nutrient uptake and synthesis of proteins, nucleotides and lipids while TORC2 regulates actin cytoskeleton and ceramide (sphingolipid) synthesis. TORC1, but not TORC2, is inhibited by the natural drug rapamycin [8, 9].

In addition to these biochemical systems, cell growth is also regulated by biophysical systems energizing nutrient and K^+ uptake and extruding protons for pH homeostasis [10-12]. Cross-talk between both kinds of systems is an important aspect of growth regulation that has been little investigated [12, 13]. In animal cells the Na^+/H^+ exchanger NHE1 is an important proton extrusion system and its activity is regulated by several protein kinases activated by growth factors [14]. The primary pump of these cells, the ouabain-sensitive Na^+,K^+-ATPase, is essential for cell growth [11, 15] and it drives nutrient uptake and Na^+/H^+ exchange [11]. This ATPase is activated by mitogens [16] but the mechanisms are mostly unknown.

The yeast *Saccharomyces cerevisiae* is a convenient model system for the study of cell cycle control and cancer [17] and we have investigated the regulation of its plasma membrane H^+-ATPase (Pma1) by the TOR protein kinase. Pma1 is a proton pump essential for cell growth [18] and activated by glucose metabolism [19]. It generates an electrochemical proton gradient that energizes nutrient uptake and regulates extracellular and intracellular pH [20].

Our results indicate that TORC1 is required for full activity of Pma1 and this regulation is mediated by the protein phosphatase Sit4, a known effector of TORC1. Also,

TORC1 subunit Tco89 activates K^+ transport. Therefore, some of the growth-activating effects of TORC1 may be explained by activation of Pma1 and K^+ transport resulting in increased nutrient uptake and optimal intracellular pH. This mechanism may also operate in animal cells, where TORC1 could activate their primary pump, the Na^+,K^+-ATPase.

Materials and Methods

Yeast strains and cell culture

Strain SH221 is a thermosensitive TOR mutant (genotype *MAT*a *leu2-3,112 trp1 ura3 rme1 his4 HMLa ade2 his3 tor1::HIS3 tor2::ADE2/YCplac111::tor2-21^{ts}*) and strain SH100 is the corresponding TOR wild type control (genotype *MAT*a *leu2-3,112 trp1 ura3 rme1 his4 HMLa ade2 his3 tor1::HIS3 tor2::ADE2/YCplac111::TOR2)* [21]. Strain BY4741 (genotype *MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) [22] is the wild type control for the null mutants *fpr1Δ0*, *sit4Δ0* and *tco89Δ0* [23].

Yeast cells were grown overnight in YPD medium (24) supplemented with 30 µg/ml adenine and incubated at either 24 or 28 ºC to early exponential phase (absorbance 0.3-0.4). Cultures were further incubated for 5 h under control or experimental conditions to reach absorbances from 1 to 3 (1.2 to 3.6 x 10^7 cells/ml), corresponding to late exponential phase. Absorbances were measured with an Ultrospec 10 of Amersham Biosciences and, when appropriate, cultures were diluted with water to obtain readings below 0.5. One unit absorbance corresponds to 1.1 mg cells/ml. Weight of cells always refers to fresh weight.

Normal YPD medium has a pH of 5.8-6.2 depending on autoclaving time. YPD medium at pH 4.1 for some experiments was made by addition (after autoclaving) 1/10 vol of 0.5 M succinic acid-Tris base pH 3.7 buffer.

Growth curves of different strains were made with a Bioscreen C (Thermo Fisher Scientific), an instruments that allows the continuous and automated recording of cell growth [25]. Saturated cultures of different strains were grown for 16-24 h to an absorbance of about 7 and used to inoculate the wells of the instrument to an initial absorbance of 0.03.

Preparation of a membrane fraction enriched in plasma membranes and determination of Pma1 activity

We have determined the activity of Pma1 in membranes isolated from growing yeast cells because glucose in the medium is a major activator of the enzyme and removal of medium results in rapid inactivation [19]. Cells were grown to late exponential phase with 50

ml YPD medium in a 250 ml flask and concentrated by centrifugation (5 min at 3000 rev/min in a Nahita 2655 centrifuge) and resuspension in 2 ml of supernatant. After 5 min incubation at growth temperature for recovery of the physiological state [19], cells were supplemented with 5 ml glass beads of 0.5 mm precooled at -20 ºC (BioSpec Products) and 400 µl of concentrated extraction buffer (0.3 M Tris-HCl pH 8.0, 0.3 M KCl and 30 mM EDTA) containing 2 µl 0.5 M dithiothreitol and 80 µl concentrated (x25) protease inhibitors cocktail (Roche Applied Science). Homogenization was effected by shaking in a vortex at top speed during 3 min. Liquid was recovered with a pipette and centrifuged during 5 min at 3000 rev/min and 4 ºC (Eppendorf R5415 R centrifuge) to remove debris. Supernatant was further centrifuged during 20 min at 13000 rev/min and 4 ºC to obtain membranes. Pellet was resuspended with a Dounce homogenizer (Fisher Scientific) in 100 µl glycerol buffer (20 % glycerol, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 1 mM dithiothreitol), diluted with 900 µl cold water and centrifuged 30 min at 13000 rev/min and 4 ºC to remove inorganic phosphate and other contaminants. The pellet was finally homogenized with 200 µl glycerol buffer and kept frozen.

Measurement of protein concentration and assay of plasma membrane ATPase (Pma1) activity have been described [26] but reactions were made in microplate wells. The ATPase assay buffer contains inhibitors of mitochondrial ATPase (azide), vacuolar ATPase (nitrate) and acid phosphatase (molybdate). Two determinations were made for every membrane sample, without and with 0.15 mM of Pma1 inhibitor orthovanadate. Pma1 activity corresponded to ATP hydrolysis resistant to azide, nitrate and molybdate and sensitive to vanadate and it represented more than 90 % of total ATP hydrolytic activity. Specific activities are given in nmoles Pi x min^{-1} x µg $protein^{-1}$.

Measurement of glucose-dependent acidification and rubidium transport

Wild type yeast (strain BY4741) and *sit4Δ* and *tco89Δ* mutants were grown in 20 ml YPD medium (100 ml flask) to late exponential phase (absorbances 1 to 3). Then cells were centrifuged 5 min at 3000 rev/min, resuspended in 20 ml water and incubated 2 h at 28 ºC to induce K^+ efflux and H^+ influx and activate in this way glucose-induced acidification and K^+ uptake [27]. After centrifugation cells were resuspended in 1 ml water.

The assay for acidification of external medium was made in 2 ml of 10 mM glycylglycine (pH 4.0) and 100 mM KCl as described [27]. Reaction was started with 50 µl 20% glucose, calibration was made with pulses of 10 µl 10 mM HCl and pH was recorded with a GLP22 pH meter (Crison). Results are expressed as nmoles H^+ x min^{-1} x mg $cells^{-1}$.

Potassium transport was measured by the uptake of its analog rubidium, which shares transporters with K^+ [28]. Cells were prepared as described above for the assay of external acidification but incubated at 20 mg/ml in a medium containing 2 % glucose and 50 mM MES (2-(N-morpholino)ethanesulfonic acid)-Tris pH 6.0. After 5 min at 24 ºC, RbCl was added to a final concentration of 5 mM and samples of 0.5 ml were taken at 0, 10 and 20 min of incubation with shaking at 24 ºC. After dilution with 10 ml cold 10 mM $MgCl_2$ cells were centrifuged 5 min at 3000 rev/min, resuspended with 10 ml cold 10 mM $MgCl_2$ and centrifuged again. Finally cells were extracted during 5 min at 95 ºC with 200 µl 10 mM $MgCl_2$ centrifuged and diluted in water for assay with an atomic absorption spectrophotometer (SENSAA of GBC Scientific Equipment) working in emission mode.

Determination of intracellular pH and potassium in yeast cells

Intracellular pH (pHi) was measured by the distribution of ^{14}C-propionic acid inside and outside cells [10, 29]. Wild type yeast (strain BY4741) and *sit4Δ* and *tco89Δ* mutants were grown in 15 ml YPD medium pH 4.1 (100 ml flask) to late exponential phase (absorbances 1 to 3). Cells were centrifuged (5 min at 3000 rev/min) and resuspended with 3 ml of supernatant. Then 3 µl ^{14}C-propionic acid (100 µCi/ml and 50-60 Ci/mol; American Radiolabeled Chemicals) were added and incubated for 10 min to equilibrate with the pH gradient. Samples of 0.5 ml were taken, diluted with 10 ml cold washing solution (20 mM KCl and 1 mM $MgCl_2$), filtered on glass fiber discs (Whatman GF/C) and washed on the filter with 10 ml cold washing solution. Filters were dried at room temperature for 30 min and radioactivity determined by liquid scintillation counting. Washing controls were made with cells treated 10 min at 95 ºC before incubation with ^{14}C-propionic acid and had radioactivity values 5-10 % of experimental samples.

For the determination of intracellular K^+ cells were grown to late exponential phase in YPD medium and cells from 5 ml culture were centrifuged 5 min at 3000 rev/min, resuspended with 5 ml cold 10 mM $MgCl_2$ and centrifuged again to remove external K^+. This washing step was repeated and finally cells were extracted during 5 min at 95 ºC with 500 µl 10 mM $MgCl_2$. After centrifugation as before, the supernatant was diluted in water for assay with an atomic absorption spectrophotometer (SENSAA of GBC Scientific Equipment) working in emission mode.

Western blot analysis of Pma1

Polyacrylamide gel electrophoresis (8% resolving gel) with sodium dodecylsulfate [30] and transfer of proteins from gel to PVDF membrane [31] were as described. Samples for electroforesis could not be prepared by the usual procedure of boiling in SDS because this aggregates Pma1. As incubation with SDS at low temperature triggers proteolysis by endogenous proteases, samples were first precipitated with 5 % trichloroacetic acid, washed with cold water and resuspended with Laemmli sample buffer at 37 ºC. Size standard was the PageRuler prestained Protein Ladder (Thermo Scientific) and the apparent molecular weights of these bands were slightly lower than theoretical. Quantification of Pma1 by western blotting was made with a rabbit polyclonal antibody against purified Pma1 [18]. The activation state of Pma1 was investigated with an antibody against the phosphorylated peptide corresponding to amino acids 904-917 of Pma1 and containing phosphoserine and phosphothreonine at positions 911 and 912, respectively [32]. Detection of the labelled blots was done using an Amersham ECL system (GE Heathcare Life Sciences, Buckinghamshire, UK) and quantification was done with the Java-based image-processing program ImageJ (http://rsb.info.nih.gov/ij/). Blots were first decorated with the antibody against the phosphorylated peptide described above, stripped by incubation for 1 h in 0.2 M glycine/HCl (pH 2.7), decorated with antibody against purified, and finally stained for total protein with Direct Blue 71.

Results

TOR is required for full activity of Pma1

TOR is an essential protein and a null mutant is non-viable. Therefore we have utilized a thermosensitive mutant generated in the laboratory of Michael N. Hall (Basel) by the following strategy [21]. In yeast there are two TOR genes, *TOR1* and *TOR2*, the Tor2 protein can form part of the two TOR complexes (TORC1 and TORC2) but Tor1 only participates in TORC1. Accordingly, *TOR2* is an essential gene bur *TOR1* is not. Strain SH221 contains a null mutation in *TOR1* and a thermosensitive mutation in *TOR2* while the control or wild type strain (SH100) contains a null mutation in *TOR1* but a wild type *TOR2* gene. As indicated in Figure 1A, at 25 ºC the thermosensitive strain grows slightly less than the control strain and reachs the same final absorbance. At 37 ºC, however, the thermosensitive strain experiences a considerable decrease in growth and reaches much lower absorbance than the control strain, that grows normally at this temperature.

As indicated in Figure 1B, in the control strain the activity of Pma1 from cells grown at 37 ℃ is 95 % of that from cells grown at 25 ℃ and the difference is not statistically significant. On the hand, in the thermosensitive mutant the activity of Pma1 from cells grown at 37 ℃ is 53 % of that from cells grown at 25 ℃ and the difference is statistically significant. Therefore, thermal inactivation of TOR decreases Pma1 activity by about half.

TORC1 regulates Pma1

As the mutant utilized for the above experiments is thermosensitive for both TOR complexes (TORC1 and TORC2) we have tested the effect of rapamycin, a specific inhibitor of TORC1 [7, 8], on Pma1 activity. In order to control for non-specific effects of the drug we used a mutant (*fpr1Δ*) devoid of the rapamycin receptor and therefore resistant to specific effects of the drug [33, 34]. As indicated in Figure 2A rapamycin at 0.1 µM strongly inhibits growth in the wild type strain but no inhibition is observed in the *fpr1Δ* mutant. Similar results were obtained when the concentration of rapamycin was raised to 0.5 µM (data not shown).

Rapamycin has no effect on the Pma1 activity of the *fpr1Δ* mutant but it reduces the activity of wild type to 57 % of control cells, a similar inhibition to the one observed with the thermosensitive TOR mutant at non-permissive temperature (see above). These results indicate that TORC1 is the TOR complex required for maximum activity of Pma1.

Sit4 mediates the effect of TORC1 on Pma1

Pma1 is a very abundant protein located at the plasma membrane [35] and its activation by TORC1 probably requires intermediate regulatory proteins because of the low abundance and poor mobility of the TOR regulatory complex [7-9]. The best-known proximal effectors of TORC1 are protein kinase Sch9, protein phosphatases 2A Pph21, PPG1 and Sit4 and Tap42, a regulatory protein interacting with these phosphatases [7-9]. *TAP42* is an essential gene [23, 36] and the phenotypes of null mutants of the other effectors indicate that *sch9Δ* [37], *pph21Δ* [38] and *ppg1Δ* [39] are acid tolerant while *sit4Δ* is acid sensitive [40]. A mutant with reduced activity of Pma1 should be acid sensitive [10, 29] and, accordingly, Pma1 activity in isolated membranes of the *sit4Δ* mutant is 55 % of wild type (Figure 3A). The Pma1 activity of *sit4Δ* is insensitive to rapamycin (less than 5 % inhibition in three biological repetitions, data not shown), suggesting that Sit4 mediates the effect of TORC1 on Pma1. Growth of *sit4Δ* cells is inhibited by rapamycin (Figure S1) because TORC1 has many

important growth promoting activities in addition to activate Pma1 [6, 7]. The rate of *in vitro* ATP hydrolysis of several Pma1 mutants has been shown to correlate with the acidification rate of glucose metabolizing cells [41]. As indicated in Figure 3B, the cells of the *sit4Δ* mutant have reduced proton extrusion activity (47 % of wild type).

Tco89, a TORC1 subunit, regulates proton efflux from cells by activating K$^+$ transport

Tco89 is the only non-essential subunit of TORC1 [7] and our results indicate that its null mutation (*tco89Δ*) has no significant effect on Pma1 activity of isolated membranes (Figure 3A). Surprisingly, this mutant exhibits a reduced acidification rate by cells, similar to *sit4Δ* mutant (Figure 3B). H$^+$ efflux from cells depends not only on proton pumping by Pma1 but also on electrical balance by cation uptake and anion efflux. In yeast the major contribution for this balance corresponds to K$^+$ transport [42, 43] and therefore we have investigated if Tco89 affects this system.

The results of Table 1 indicate that the rate of rubidium uptake (utilized as a tracer of K$^+$ uptake [28]) in the *sit4Δ* and *tco89Δ* mutants is 51 and 53 % of wild type, respectively. As indicated in the same table, this reduction in uptake rate correlates with a decrease in the steady-state level of intracellular K$^+$ in growing cells, which in the *sit4Δ* and *tco89Δ* mutants is 61 and 65 % of wild type, respectively. We can interpret these results with a model where Sit4 and Tco89 regulate proton efflux from yeast cells by different mechanisms: Sit4 activates directly Pma1, the proton pump, and indirectly K$^+$ transport (through hyperpolarization) while Tco89 directly activates K$^+$ transport and indirectly proton efflux from cells (through depolarization), without direct effects on Pma1.

Intracellular pH of *sit4Δ* and *tco89Δ* mutants is more acidic than in wild type

The intracellular pH of *sit4Δ* and *tco89Δ* mutants should be more acidic than in wild type because of their reduced capability for proton efflux (Figure 3B). This was confirmed in Figure 4. For wild type cells growing at pH 4.1 pHi was 6.42, similar to previously described [10], and addition of acetic acid reduced this value to 6.20. The pHi of growing cells of *sit4Δ* and *tco89Δ* mutants was 6.25 and 6.27, respectively, and decreased to 6.05 and 6.03 in the presence of acetic acid.

Growth of *sit4Δ* and *tco89Δ* mutants is sensitive to weak organic acids

The decrease in H^+ efflux, K^+ transport and pHi of *sit4Δ* and *tco89Δ* mutants described above suggest that growth of these strains should be slower than wild type [10] and more sensitive to weak organic acids that diffuse into cells to lower pHi [40]. Actually, both mutants are known to have reduced growth [44] and our results provide a mechanism for this observation based on pHi and K^+ homeostasis. Concerning organic acids, *sit4Δ* has been described to be sensitive to sorbic acid [40] but there is no information about *tco89Δ*. Growth experiments were performed in a system (Bioscreen C) that continuously register growth and allows the calculation of growth parameters (lag, growth rate, yield) with greater accuracy than previous methods.

As indicated in Figure 5 A and Table S1, in normal growth medium *sit4Δ* and *tco89Δ* have increased lag (2.5 h in both mutants versus 1.5 h in wild type) and decreased growth rate (70 and 86 % of wild type, respectively). Both mutants are more sensitive to sorbic and acetic acids than wild type (Figure 5 B and C), with a greater increase in lag and a greater reduction in growth rate. Growth parameters in the presence of acids were referred to the values for the same strain without acids (Table S1). Sorbic and acetic acids decrease growth rate in the control strain by 30 and 45 % and in the *sit4Δ* mutant by 50 and 55 %, respectively. Concerning the lag, sorbic and acetic acids increase it 1.8-fold in wild type but 2.4- and 4.6-fold, respectively, in *sit4Δ*. *tco89Δ* was more acid-sensitive than *sit4Δ* despite having less growth defect in normal medium. For example, sorbic and acetic acids decrease growth rate by 70 % and 80 %, respectively.

Discussion

Our results indicate that growth regulation by TORC1 includes basic mechanisms of pH and K^+ homeostasis. Cell growth requires high intracellular pH and K^+ [10-12] and the activation of H^+ extrusion and K^+ uptake by TORC1 described in the present work is an important function of TORC1 mediated by Sit4 and Tco89, respectively. Sit4 is a proximal TORC1 effector and Tco89 a subunit of the complex [7].

Sit4 belongs to a group of type 2A protein phosphatases inhibited by TORC1 [7-9] by promoting binding of the inhibitory subunit Tap42 [36, 45]. Sit4 dephosphorylates and activates protein kinase Npr1 and transcription factor Gln3, both involved in scavenging secondary nitrogen sources [46, 47]. However, a mechanism where TORC1 inhibits Sit4 and this phosphatase were inhibitory for Pma1 is not supported by our results. If this were the case

the *sit4Δ* mutant should have high Pma1 activity and we found the contrary: this mutant has low Pma1 activity insensitive to rapamycin, as if Sit4 were an activator of Pma1 mediating the positive effect of TORC1 on this proton pump. A plausible mechanism would be that, in the context of the proton pump, TORC1 activates Sit4 and this phosphatase activates Pma1. This could occur by direct dephosphorylation of Pma1 or by indirect modification of the proton pump after dephosphorylation of some intermediate regulator.

Glucose metabolism is the major factor activating Pma1 [19] and the mechanism involves double phosphorylation of the enzyme at Ser911 and Thr912 by some unknown mechanism [32, 48] as the most important modification. As indicated at Figure S2, neither rapamycin nor the *sit4Δ* mutation decrease this important phosphorylation. Other phosphorylations affecting Pma1 activity to a lower extent occur at Ser899 [32] and Ser507 [49]. The last one deserved special attention because it is inhibitory and a phosphatase acting on phosphorylated Ser507 would activate Pma1.

It has recently been described that rapamycin decreases K^+ accumulation in yeast cells independently of the Trk1 and Trk2 high affinity transporters [50]. Our results suggest that this effect could be mediated by the effect of TORC1 on Pma1 because inhibition of the pump would result in membrane depolarization and decreased K^+ uptake mediated by an uncharacterized low-affinity transporter [51]. On the other hand, our finding that TORC1 subunit Tco89 activates K^+ transport without affecting Pma1 activity suggest that this subunit of unknown function is an effector of TOR specific for activation of K^+ transporters by unknown mechanism.

One important aspect of these findings is that there is a reciprocal regulation between TORC1 and the H^+ and K^+ transport systems. This was first described for the TORC1-K^+ pair, where a decrease in K^+ accumulation activates TORC1 and inactivation of TORC1 decreases K^+ accumulation [50]. In the case of H^+ transport it has been described that activation of Pma1 by glucose increases TORC1 activity and that this effect is mediated by an increase in intracellular pH [52]. Our results show the reciprocal regulation, TORC1 regulates Pma1.

As a final consideration, it must be pinpointed that our work has uncovered a connection between two essential regulators of cell growth, TORC1 and Pma1, and that this add a novel perspective to the central problem of growth control. For example we predict that this regulation of the proton pump by TORC1 would be general in organisms with a chemiosmotic circuit of protons (such as fungi and plants) and that in animal cells, with a chemiosmotic circuit of sodium, TORC1 will regulate the sodium-potassium pumping ATPase.

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Author contributions

S.M., M.D.P., M.C. and C.T. determined Pma1 activity and cell acidification assays; A.R. measured intracellular pH,; A.R., M.C. and S.C.S. determined rubidium uptake and intracellular K^+; C.M., M.C. and A.R. performed growth experiments with Bioscreen C; JMM and RS planed the experiments, discussed results and wrote the manuscript.

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Table 1. Altered K$^+$ homeostasis in *sit4Δ* and *tco89Δ* mutants

Rubidium uptake was used as tracer for K$^+$ uptake in cells starved in water as described in methods to partially deplete internal K$^+$. Intracellular K$^+$ was determined in growing cells at late exponential phase. Results are the average of three experiments with its standard error.

Strain	Rubidium uptake rate nmoles / mg cells x 10 min	Intracellular K$^+$ levels nmoles/mg cells
BY4741 wild type	1.37 ± 0.02	68.0 ± 2.5
sit4Δ mutant	0.70 ± 0.01	41.5 ± 1.2
tco89Δ mutant	0.73 ± 0.13	44.5 ± 1.5
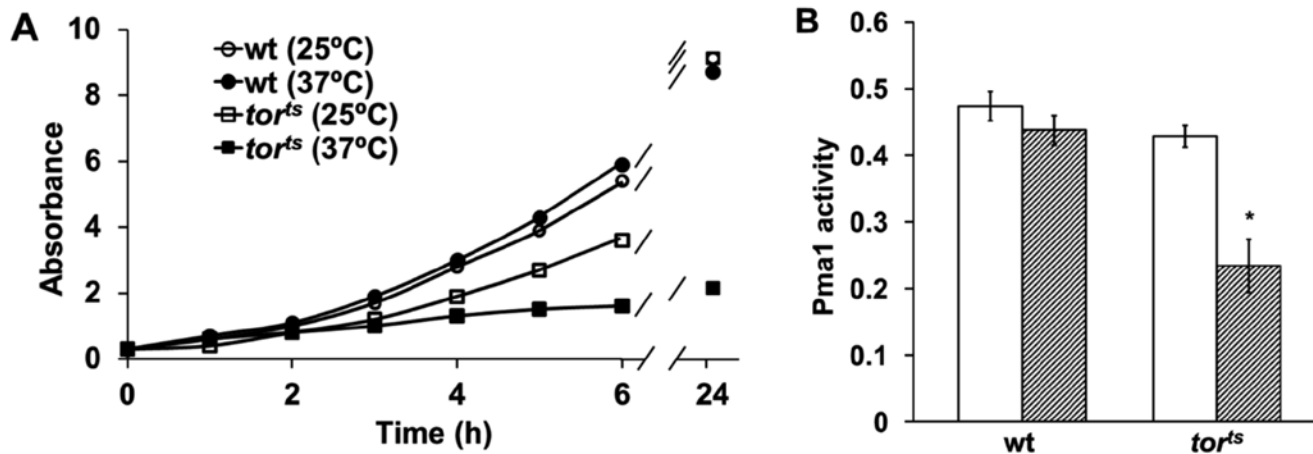
Figure 1. Inhibition of TOR in a thermosensitive mutant decreases Pma1 activity. A: typical growth of wild type yeast (strain SH100, wt, circles) and thermosensitive mutant (strain SH221; *tor^ts*, squares) at either 25 ℃ (open symbols) or 37 ℃ (filled symbols) in YPD medium. B: Pma1 activity determined in membranes isolated from cells (wild type and *tor^ts* mutant) grown for 5 h at either 25 ℃ (open bars) or 37 ℃ (filled bars) in YPD medium. Pma1 activity is expressed in nmoles Pi x min^{-1} x µg protein^{-1}. The experiment has been repeated three times (3 biological repetitions) and error bars correspond to standard errors. Asterisk above a filled bar indicates a significant difference ($p < 0.05$, Student's t-test) with control (adjacent open bar).
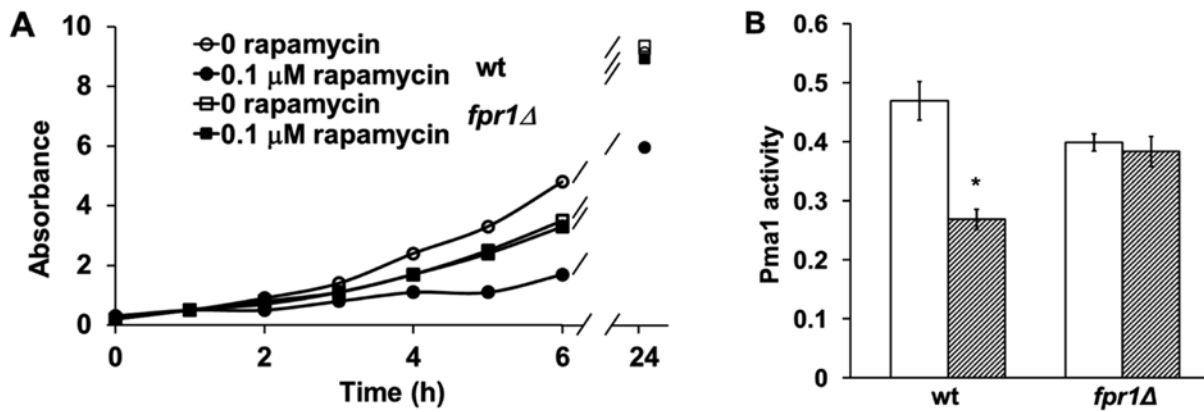
Figure 2. Rapamycin, a TORC1 inhibitor, decreases Pma1 activity in a manner dependent on rapamycin receptor Fpr1. A: growth of wild type yeast (strain BY4741, wt, circles) and *fpr1Δ* mutant (squares) in the absence of rapamycin (open symbols) and with 0.1 μM rapamycin (filled symbols) in YPD medium. B: Pma1 activity determined in membranes isolated from cells (wild type and *fpr1Δ* mutant) grown for 5 h in the absence (open bars) or presence (filled symbols) of 0.1 μM rapamycin in YPD medium. Pma1 activity is expressed in nmoles Pi x min^{-1} x μg protein^{-1}. All experiments have been repeated three times (3 biological repetitions) and error bars correspond to standard errors. Asterisk above a filled bar indicates a significant difference ($p < 0.05$, Student's t-test) with control (adjacent open bar).
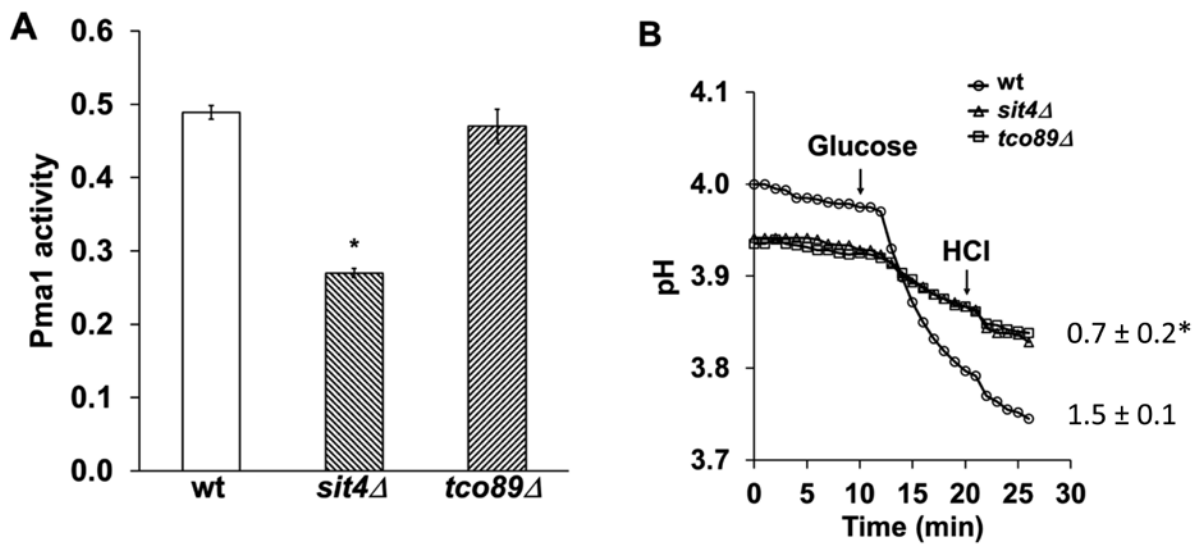
Figure 3. Effect of null mutations in TORC1 effector Sit4 and TORC1 subunit Tco89 on Pma1 activity and proton efflux from cells. (A) Pma1 activity in isolated membranes from control strain (BY4741, wt), *sit4Δ* and *tco89Δ* mutants growing on YPD medium. Ordinate units are nmoles Pi x min^{-1} x µg protein^{-1}. (B) Glucose-dependent acidification of external medium in cells from wild type (control strain BY4741; circles), *sit4Δ* (triangles) and *tco89Δ* (squares) mutants. Initial rates of acidification (expressed as nmoles H$^+$ x min^{-1} x mg cells^{-1}) are indicated at the right. All experiments have been repeated three times (3 biological repetitions) and error bars (in A) and "± values" (in B) correspond to standard errors. Asterisk indicates a significant difference ($p < 0.05$, Student's t-test) with control.
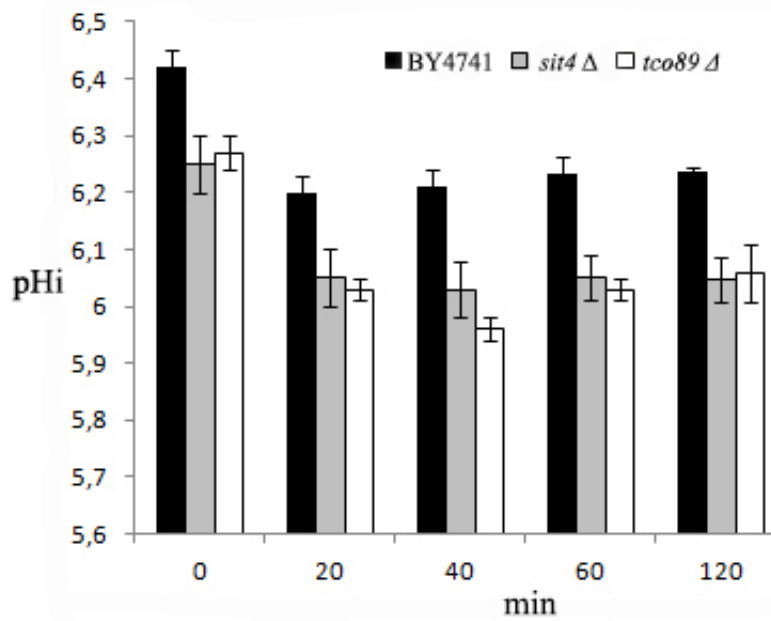
Figure 4. Intracellular pH of *sit4Δ* and *tco89Δ* mutants is more acidic than in wild type. Intracellular pH was measured in growing cultures al late exponential phase by the distribution of ^{14}C-propionic acid as described in Methods. Time 0 corresponds to the culture before addition of acetic acid at 60 mM to induce intracellular acidification. Times 20, 40, 60 and 120 min correspond to incubation times in the presence of acetic acid. The experiment has been repeated three times (3 biological repetitions) and error bars correspond to standard errors. All values of *sit4Δ* and *tco89Δ* mutants have a significant difference ($p < 0.05$, Student's t-test) with control (BY4741).
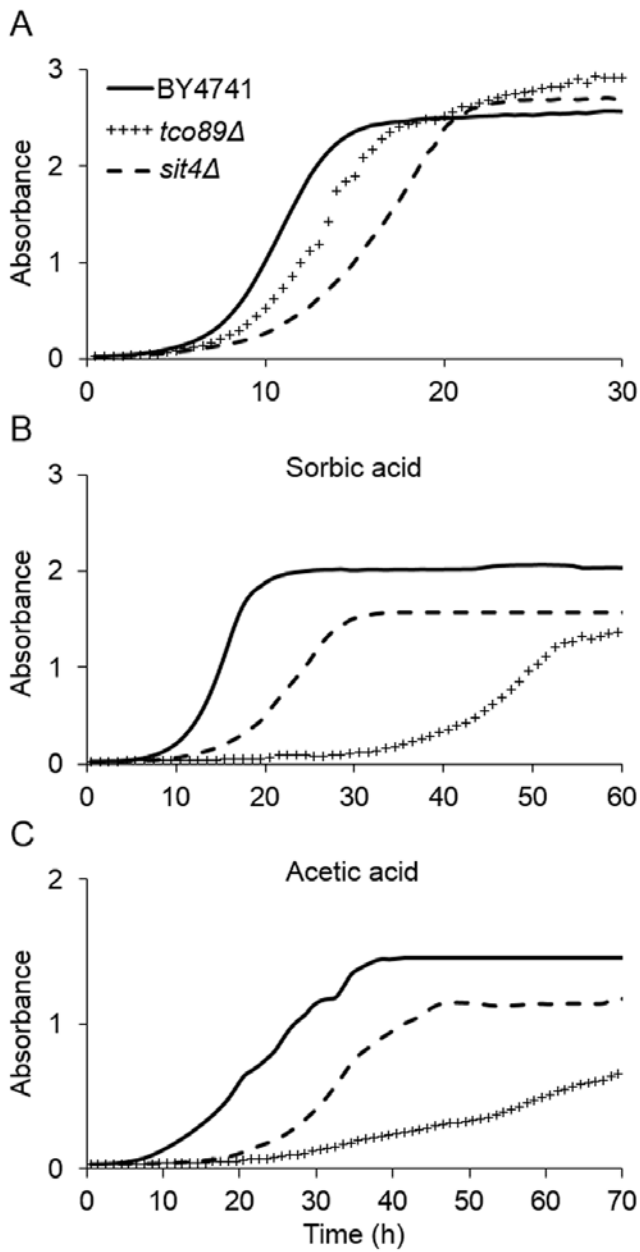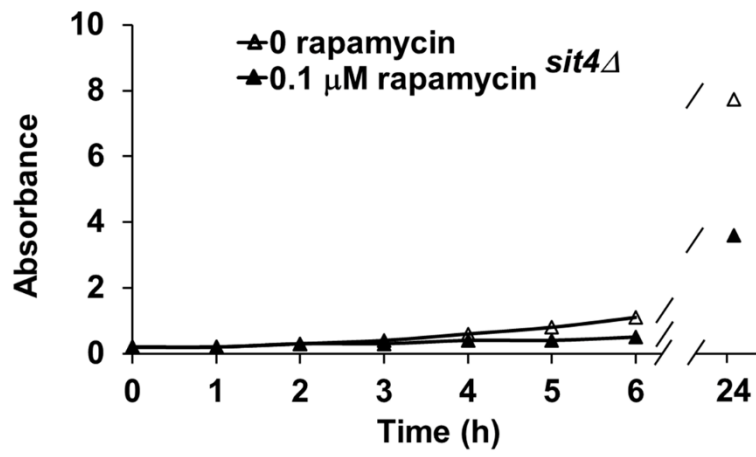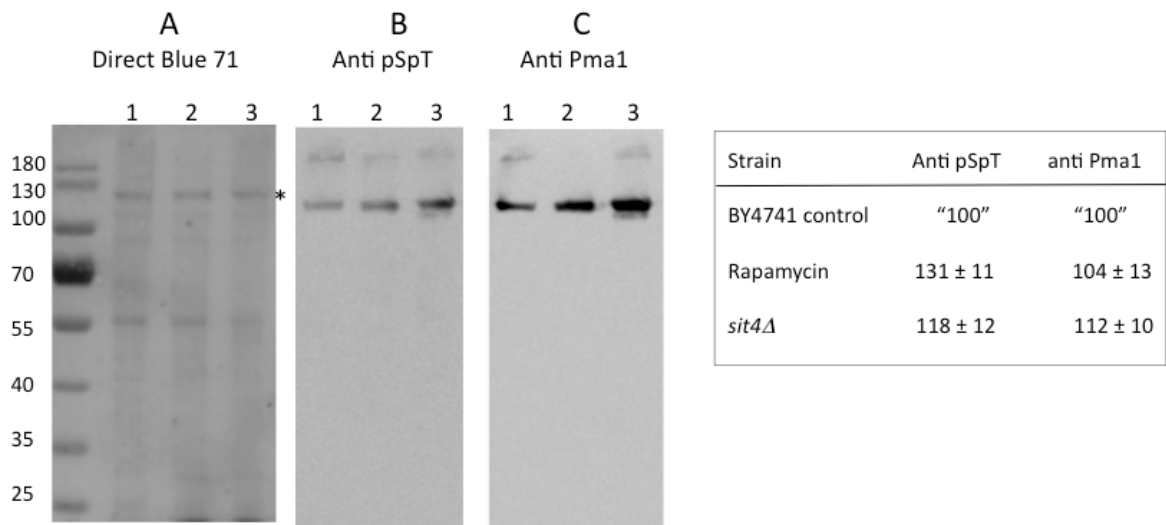
Figure 5. Growth of BY4741 and *sit4Δ* and *tco89Δ* mutants in YPD medium pH 4.1 (A) and in this medium supplemented with 1.5 mM sorbic acid (B) and 60 mM acetic acid (C). A typical experiment is shown and the average growth parameters of three biological repetitions are shown in Table S1.

Supplementary Figure S1. Growth of *sit4Δ* mutant is inhibited by rapamycin.

A	B	C
Direct Blue 71	Anti pSpT	Anti Pma1

Strain	Anti pSpT	anti Pma1
BY4741 control	"100"	"100"
Rapamycin	131 ± 11	104 ± 13
sit4Δ	118 ± 12	112 ± 10

Supplementary Figure S2. Rapamycin treatment and the *sit4Δ* mutation slightly increase the phosphorylation of the major activation site of Pma1. A: staining of blotted proteins by Direct Blue 71. First lane corresponds to size standards and the molecular weights of the bands are indicated in kDa. B: immunodetection of phosphorylation of the major activation site of Pma1, the double phosphorylation in Ser911 and Thr912 (Anti pSpT). C: immunodetection of Pma1 protein. In all panels lanes 1 correspond to membranes from strain BY4741 grown under normal conditions, lane 2 to membranes from BY4741 treated with rapamycin and lane 3 to membranes from the *sit4Δ* mutant. Results of a typical experiment are shown. Statistical data after densitometry (Image J) of three experiments are shown in the table. To correct for differences in loading of the lanes, the signal of the double phosphorylation in Ser911 and Thr912 and of Pma1 protein were normalized with the signal of a protein band in panel A with molecular weight about 120 kDa (just above the Pma1 band, see asterisk). Values in the table are relative to BY4741 taken as 100% and correspond to the average ± standard error.

Supplementary Table S1. Growth parameters of control strain (BY4741) and *sit4Δ* and *tco89Δ* mutants in the presence of weak organic acids. Absolute values are given for every strain in the first column (YPD medium adjusted to pH 4.1 and no more additions). In the columns corresponding to media supplemented with acetic or sorbic acids, values for each strain are relative to those of the same strain in the first column. Results are the average of three experiments like the one shown in Figure 4. The standard error ranged from 1/20 to 1/10 of the average and is not depicted for clarity.

	YPD pH 4.1			+ acetic acid (60 mM)			+ sorbic acid (1.5 mM)		
Strain	lag h	rate h⁻¹	yield absorb.	lag (% of YPD pH 4.1)	rate	yield	lag (% of YPD pH 4.1)	rate	yield
BY4741	1.5	0.44	2.8	185	55	65	180	72	75
sit4Δ	2.5	0.31	2.9	457	45	41	240	52	62
tco89Δ	2.5	0.38	3.1	440	21	23	240	31	45