State Transitions in the TORC1 Signaling Pathway and Information Processing in *Saccharomyces cerevisiae*

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ABSTRACT TOR kinase complex I (TORC1) is a key regulator of cell growth and metabolism in all eukaryotes. Previous studies in yeast have shown that three GTPases—*Gtr1*, *Gtr2*, and *Rho1*—bind to TORC1 in nitrogen and amino acid starvation conditions to block phosphorylation of the S6 kinase *Sch9* and activate protein phosphatase 2A (PP2A). This leads to downregulation of 450 *Sch9*-dependent protein and ribosome synthesis genes and upregulation of 100 PP2A-dependent nitrogen assimilation and amino acid synthesis genes. Here, using bandshift assays and microarray measurements, we show that the TORC1 pathway also populates three other stress/starvation states. First, in glucose starvation conditions, the AMP-activated protein kinase (AMPK/*Snf1*) and at least one other factor push the TORC1 pathway into an off state, in which *Sch9*-branch signaling and PP2A-branch signaling are both inhibited. Remarkably, the TORC1 pathway remains in the glucose starvation (PP2A inhibited) state even when cells are simultaneously starved for nitrogen and glucose. Second, in osmotic stress, the MAPK *Hog1*/p38 drives the TORC1 pathway into a different state, in which *Sch9* signaling and PP2A-branch signaling are inhibited, but PP2A-branch signaling can still be activated by nitrogen starvation. Third, in oxidative stress and heat stress, TORC1-*Sch9* signaling is blocked while weak PP2A-branch signaling occurs. Together, our data show that the TORC1 pathway acts as an information-processing hub, activating different genes in different conditions to ensure that available energy is allocated to drive growth, amino acid synthesis, or a stress response, depending on the needs of the cell.

THE growth rate of a eukaryotic cell is controlled by a complex network of signaling pathways and transcription factors. At the heart of this network lies the TOR kinase, acting as part of TOR complex I (TORC1). This 2-MDa complex responds to rapamycin, nutrient, stress, and hormone signals (Heitman *et al.* 1991; Zheng *et al.* 1995; Cardenas *et al.* 1999; Inoki *et al.* 2002; Loewith *et al.* 2002; Urban *et al.* 2007; Loewith and Hall 2011; Zoncu *et al.* 2011) and in turn phosphorylates numerous proteins to control translation, ribosome synthesis, autophagy, and a variety of metabolic pathways (Barbet *et al.* 1996; Cardenas *et al.* 1999; Powers and Walter 1999; Crespo *et al.* 2001; Martin *et al.* 2004; Huber *et al.* 2009; Yorimitsu *et al.* 2009; Kamada *et al.* 2010; Hsu *et al.* 2011; Loewith and Hall 2011; Zoncu *et al.* 2011).

Experiments in yeast have shed light on the structure and function of the TORC1 pathway (Figure 1A) and help explain its complexity. Specifically, it is known that when cells are grown in rich medium, TORC1 activates the S6 kinase *Sch9*, as well as several transcription factors (including *Sfp1*, *Dot6/Tod6*, *Fhl1*, *Maf1*, and *Stb3*; Figure 1A), to drive protein and ribosome synthesis (Upadhyaya *et al.* 2002; Jorgensen *et al.* 2004; Marion *et al.* 2004; Schawalter *et al.* 2004; Liko *et al.* 2007; Urban *et al.* 2007; Huber *et al.* 2009; Lee *et al.* 2009; Lempiainen *et al.* 2009; Lippman and Broach 2009; Huber *et al.* 2011). At the same time, active TORC1 binds and represses Tap42-PP2A, an activator of (i) the amino acid synthesis and nitrogen assimilation pathways via *Npr1*, *Gln3*, *Rtg1/Rtg3*, and other factors; (ii) the environmental stress response via *Msn2/Msn4*; and (iii) autophagy via *Atg1* and/or other factors (Figure 1A) (Di Como and Arndt 1996; Beck and Hall 1999; Crespo *et al.* 2002; Duvel *et al.* 2003; Santhanam *et al.* 2004; Yan *et al.* 2006; Shin *et al.* 2009; Yorimitsu *et al.* 2009; Breitkreutz *et al.* 2010; Huber *et al.* 2011). In contrast, when cells are starved for nitrogen or treated with rapamycin, TORC1 is inhibited so that protein/ribosome synthesis halts while the cell synthesizes glutamine

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and other amino acids (Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999; De Craene *et al.* 2001; Crespo *et al.* 2002; Duvel *et al.* 2003; Huber *et al.* 2009; Loewith and Hall 2011). Altering the nitrogen source in the growth medium, or blocking glutamine synthesis using a drug, also activates signaling through portions of the protein phosphatase 2A (PP2A) branch of the pathway (Crespo *et al.* 2002; Georis *et al.* 2011; Rai *et al.* 2013). These results, and others, have led to the conclusion that the TORC1 pathway acts primarily as a nitrogen/amino acid starvation response circuit (Broach 2012).

While the TORC1 pathway clearly plays an important role in nitrogen and amino acid signaling, TORC1 is also thought to respond to a variety of other stress and starvation signals, including osmotic, heat, and oxidative stress, as well as carbon and phosphate starvation (Urban *et al.* 2007; Loewith and Hall 2011). However, it remains unclear how these additional stress/starvation conditions influence the global output of the TORC1 pathway since experiments carried out to date have followed the regulation of only one or a few proteins (e.g., *Sfp1* and *Sch9*) (Marion *et al.* 2004; Urban *et al.* 2007).

Here, to investigate how the TORC1 pathway processes information from different inputs, we use DNA microarrays and bandshift assays to measure *Sch9*-branch and PP2A-branch signaling in a wide variety of stress and starvation conditions. Remarkably, we find that heat stress, osmotic stress, oxidative stress, and glucose starvation all push the TORC1 pathway into a previously unknown state, in which signaling through the *Sch9* branch of the pathway is inactivated, but the PP2A branch of the pathway remains repressed. Furthermore, we find that even in cells simultaneously starved for nitrogen and glucose, the PP2A branch of the TORC1 pathway remains in a low-activity state. Thus, all stress and starvation conditions trigger inhibition of protein/ribosome synthesis through the *Sch9* branch of the TORC1 pathway, but only nitrogen starvation in the presence of glucose (energy) triggers robust activation of nitrogen assimilation and amino acid synthesis pathways through the PP2A branch of the TORC1 pathway.

To probe the mechanisms underlying the condition-dependent output of the TORC1 pathway we also examined the influence that the known nitrogen/amino acid response regulators—the Rag proteins *Gtr1/Gtr2* (Sancak *et al.* 2008; Binda *et al.* 2009), the small GTPase *Rho1* (Yan *et al.* 2012), and the SEA-associated proteins *Npr2/Npr3* (Neklesa and Davis 2009; Panchaud *et al.* 2013)—have on TORC1 pathway signaling in different conditions. We find that these proteins, known to bind TORC1 at the vacuolar membrane, and in the case of *Rho1* to alter its interaction with Tap42-PP2A (Sancak *et al.* 2008, 2010; Binda *et al.* 2009; Yan *et al.* 2012), primarily influence the TORC1 pathway in nitrogen/amino acid starvation conditions. We also demonstrate that the AMP-activated protein kinase (AMPK/*Snf1*) inhibits signaling through the TORC1 pathway in glucose starvation conditions, while the MAPK *Hog1*/p38 inhibits signaling

through the TORC1 pathway in osmotic stress. Thus, different signaling pathways drive the TORC1 pathway into each of its signaling states.

Taken together, our data show that the TORC1 pathway acts as an information-processing hub, activating different genes in different conditions to ensure that available energy is allocated to drive growth, amino acid synthesis, or a stress response, depending on the needs of the cell. These results reveal the underlying design principles of the evolutionarily conserved TORC1 circuit and serve as an important starting point for building a complete model of the TORC1 pathway and cell growth control.

Materials and Methods

Saccharomyces cerevisiae strains

All strains used in this study were generated from diploid *Saccharomyces cerevisiae*, W303 strain background (*trp1*, *can1*, *leu2*, *his3*, *ura3*), except Dot6-YFP, Tod6-YFP, and *Sfp1*-YFP, which were made in haploid cells, as described in Supporting Information, Table S3.

Gene expression microarray experiments

We used an overnight culture of ACY044 [wild-type (wt) strain], ACY509, or ACY142 to inoculate a 1-liter culture to an OD₆₀₀ of 0.1 (in either YEPD or SD medium, as appropriate) in a 2.8-liter conical flask shaking at 200 rpm at 30°. We grew these cells to an OD₆₀₀ between 0.55 and 0.60 and then collected 250 ml of cells by filtration and froze them in liquid nitrogen. At this point the remaining cells were subjected to stress (by addition of KCl in YEPD, addition of hot YEPD and incubation at 42°, or addition of H₂O₂); treated with 200 ng/liter rapamycin or 100 nM 1-NM-PP1; or captured on a filter and washed with SD medium missing amino acids, nitrogen and amino acids, or glucose. Cells were then grown in the appropriate condition for 20 min before 250–300 ml of cells was collected by filtration and frozen in liquid nitrogen. This time point was selected as previous work shows that the peak of the transcriptional response to stress/starvation occurs 15–20 min after the application of stress (Gasch *et al.* 2000; Capaldi *et al.* 2008). RNA was then purified from the frozen cells, converted into cDNA using reverse transcription, labeled with Cy3 (prestress/starvation cells) or Cy5 (poststress/starvation cells), and examined using an Agilent microarray (Yeast V2) and an Axon 4000B scanner (Capaldi *et al.* 2008; Capaldi 2010). The average intensity of the Cy3 and Cy5 fluorescence at each spot was then extracted using Genepix 7 (Molecular Devices), and the data were loaded into the Stanford Microarray Database (SMD). The data were then exported from the SMD database, using a filter to eliminate spots where both the Cy5 and Cy3 signals are <1.5-fold above the background signal (defined by the average Cy5 and Cy3 signals on the negative control spots).

Figure 1 Structure and function of the TORC1-dependent transcriptional network. (A) TORC1 acts through numerous downstream effector proteins to regulate protein and ribosome synthesis and a range of metabolic pathways (Martin *et al.* 2004; Wullschlegel *et al.* 2006; Lempiainen *et al.* 2009; Sengupta *et al.* 2010; Huber *et al.* 2011; Loewith and Hall 2011). The metabolic pathways are, to a large degree, regulated by the Tap42/PP2A protein phosphatases (Beck and Hall 1999; Jacinto *et al.* 2001; Duvel *et al.* 2003; Santhanam *et al.* 2004; Huber *et al.* 2009). The activity of the PP2A complex, which includes the regulator Tap42, depends on both a physical interaction with and phosphorylation by TORC1 (Jiang and Broach 1999; Yan *et al.* 2006, 2012). Kinases are shown in blue, transcription factors in red, and phosphatases in green. (B) The gene expression program activated by the TORC1 pathway depends on the cellular condition. The global gene expression program activated in 200 ng/liter rapamycin, complete nitrogen starvation (no ammonium sulfate or amino acids), amino acid starvation, glucose starvation, 0.4 M KCl stress, 2 mM H₂O₂ stress, and 42° heat stress was measured by comparing the mRNA levels before (Cy3, green) and after 20 min of stress/starvation (Cy5, red), on a two-color DNA microarray. For comparison we also show the gene expression changes that occur when all three PKA kinases (Tpk1–3) are inactivated for 20 min, using the chemical inhibitor 1-NM-PP1 (Tpk1–3^{AS} + 1-NM-PP1). Note that this last experiment was carried out in a strain carrying point mutations in Tpk1–3 (Tpk1–3^{AS}) that render them inactive in the concentration of 1-NM-PP1 used here (100 nM) (Zaman *et al.* 2009). Other gene groups regulated by TORC1 are shown in Figure S1. (C) Number of TORC1-PP2A-dependent genes, from B, induced more than three-, four-, six-, and eightfold in key stress/starvation conditions (of 101 genes total).

To prepare Figure 1B and Figure S1, the microarray data for experiments examining the response to rapamycin, nitrogen starvation, amino acid starvation, glucose starvation,

KCl stress, 42° heat stress, H₂O₂, and 1-NM-PP1 were clustered using the correlation similarity metric and centroid linkage in Cluster 3.0 (De Hoon *et al.* 2004). The resulting

cluster groups (Figure 1 and Figure S1) were then analyzed using GO Stat (Beissbarth and Speed 2004) and YEAS-TRACT (Teixeira *et al.* 2006, 2014; Monteiro *et al.* 2008; Abdulrehman *et al.* 2011) to map the function and regulation of each gene module (Table S2).

The full microarray data set for this article can be found in Table S1 (first tab) or downloaded from the GEO database, accession no. GSE58992.

Bandshift experiments

Bandshift measurements were performed using a modified version of the protocol developed by Urban and Loewith (Urban *et al.* 2007). Cultures were grown in 500-ml conical flasks shaking at 200 rpm and 30° until midlog phase (OD₆₀₀ between 0.55 and 0.6). At this point, a 47-ml sample, providing the zero time point, was collected, mixed with 3 ml 100% trichloroacetic acid (TCA), and held on ice for at least 30 min (and up to 6 hr). For rapamycin, 1-NM-PP1, osmotic stress, oxidative stress, and heat stress experiments, the remaining culture volume was adjusted to 175 ml before adding the drug alone, KCl in YEPD or SD, H₂O₂, or hot SD (resulting in 200 ng/liter rapamycin, 100 nM 1-NM-PP1, 0.375 M KCl, 2 mM H₂O₂, or 42°). For all other experiments, the remaining culture was collected by filtration, washed with 100 ml of treatment media, and transferred to a new 500-ml conical flask containing 175 ml of the treatment media. Cultures undergoing treatment were returned to shakers for further growth and then 47-ml samples were collected and treated with TCA, as before, at 5-, 10-, and 20-min time points. TCA-treated samples were centrifuged at 4000 rpm for 5 min at 4° to collect the cell pellets, which were then washed twice with 4° water and twice with acetone and disrupted by sonication at 15% amplitude for 5 sec before centrifugation at 8000 rpm for 30 sec. Cell pellets were then dried in a speedvac for 10 min at room temperature and frozen until required at −20°.

Protein extraction was performed by bead beating (6 × 1 min, full speed) in urea buffer [6 M urea, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM PMSF, 5 mM NaF, 5 mM NaN₃, 5 mM NaH₂PO₄, 5 mM p-nitrophenylphosphate, 5 mM β-glycerophosphate, 1% SDS] supplemented with complete protease and phosphatase inhibitor tablets (Roche 04693159001 and 04906845001). The lysate was collected after centrifugation for 5 min at 3000 rpm, resuspended into a homogenous slurry by vortexing, and heated at 65° for 10 min. Soluble proteins were then separated from insoluble cell debris by centrifugation at 15,000 rpm for 5 min, and the lysates were stored at −80° until required. C-terminal epitope-tagged *Sch9* samples were subjected to cleavage by 2-nitro-5-thiocyanatobenzoic acid (NTCB) for 12–16 hr at room temperature in the dark (1 mM NTCB and 100 mM CHES, pH 10.5) before further analysis. Cell extracts were then heated to 95° in SDS sample buffer for 5 min before they were run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and then detected using 12CA5 (anti-HA) or 9E10 (anti-MYC).

The proteins used to monitor Tap42/PP2A signaling (*Nnk1*, *Npr1*, and *Gln3*) were selected by running preliminary experiments to determine which PP2A targets/components [including *Ksp1*, *Gat1*, *Hrr25*, *Sit4*, *Tip41*, *Tap42*, and *Rtg1* (Schmidt *et al.* 1998; Beck and Hall 1999; Breitzkreutz *et al.* 2010; Huber *et al.* 2011)] have the clearest bandshift after rapamycin treatment. *Gln3* had a (moderate) bandshift, similar to that found for several the other targets, but was chosen since it has been used in other studies examining PP2A signaling (Cox *et al.* 2004; Tate *et al.* 2009; Yan *et al.* 2012). To ensure consistent results all subsequent bandshift experiments (including for *Sch9*, *Dot6*, *Tod6*, and the *Npr1* target *Par32*) were run at 80 V for 3 hr on a 10% acrylamide gel (*Sch9*), a 7.5% acrylamide gel (*Dot6*, *Tod6*, and *Par32*), or a 5% acrylamide gel (*Gln3*, *Npr1*, and *Nnk1*). A protein marker [Bio-Rad (Hercules, CA) Precision plus dual color] was included on each gel and used to ensure that the region of the Western blot shown in each panel (condition/mutant) is the same: 40–60 kDa for *Sch9*, 70–120 kDa for *Tod6*, 90–160 kDa for *Dot6*, 115–175 kDa for *Npr1*, 120–180 kDa for *Gln3*, and 70–130 kDa for *Par32*. For *Nnk1* the region of the gel shown includes the 110- to 170-kDa range, except in the combined conditions, where the range 125–190 kDa is shown so that the hyperphosphorylated band found at 10–20 min can be seen.

Protein mobility shifts were quantified using a custom MATLAB script. This script requires the user to define lanes of interest on the gel image. The data in each lane are then simplified, by calculating the average signal intensity at each position along the length of the lane, and normalized (so that the total signal matches that of the *t* = 0 control). These values are then used to calculate a position-weighted mean for each data series/lane. Specifically, we defined the first pixel/data point in a gel as 100% phosphorylated and the last as 0% phosphorylated. Pixels in between these points were weighted (between 100 and 0) based on their position relative to the top and bottom of the gel (on a linear scale). The values for each position (pixel) were then summed to calculate the total amount of protein phosphorylation in a sample. Finally, since these numbers are in arbitrary units, we normalized all of the values to those found in the wild-type strain under the same stress or starvation conditions. For example, for the *Sch9* data the phosphorylation values in all strains were multiplied by constant *A* and added to a constant *B*, so that the values in the wild-type strain at time = 0 min were 1.0 and time = 5 min were 0.

Results

Condition-dependent gene regulation in budding yeast

To gain insight into the input:output characteristics of the TORC1 pathway, we exposed budding yeast to a wide range of stress and starvation stimuli, including nitrogen starvation, amino acid starvation, glucose starvation, osmotic stress, heat stress, oxidative stress, and the potent TORC1 inhibitor rapamycin and measured the transcriptional responses. In

starvation experiments cells were grown to midlog phase and then transferred into medium missing amino acids, nitrogen, or glucose. In stress experiments cells were grown to midlog phase and then treated with 0.4 M KCl, 2 mM H₂O₂, or 200 ng/ml rapamycin or exposed to heat stress (42°). Finally, after 20 min of stress/starvation treatment, cells were harvested and the mRNA levels measured using DNA microarrays (see *Materials and Methods*).

We then analyzed the data in two steps: First, we identified the TORC1-dependent genes, using the rapamycin response as a benchmark. In line with previous results (Hardwick *et al.* 1999), we found that rapamycin triggers upregulation of 578 genes and downregulation of 596 genes, by twofold or more (Table S1). Second, we clustered the data for all 1174 TORC1/rapamycin-dependent genes (see *Materials and Methods*). This led to the identification of three major gene groups: (1) 101 genes involved in nitrogen and amino acid metabolism, known targets of TORC1-PP2A (Duvel *et al.* 2003) (Figure 1B; Table S2, cluster 2); (2) 450 genes involved in ribosome and protein synthesis, known targets of TORC1-Sch9 (Urban *et al.* 2007) (Figure 1B; Table S2, cluster 4); (3) 426 genes involved in carbohydrate metabolism, regulated by the general stress transcription factors *Msn2* and *Msn4* (Figure S1; Table S2, cluster 3). We dropped this last group from our analysis as *Msn2/4* are primarily controlled by factors other than TORC1, including PKA, *Hog1*, and *Snf1* (Gorner *et al.* 1998; De Wever *et al.* 2005; Capaldi *et al.* 2008; Petrenko *et al.* 2013).

Examining the expression of the TORC1-Sch9 and TORC1-PP2A-dependent gene modules revealed that only nitrogen and (to some extent) amino acid starvation activate a transcriptional response similar to that found in rapamycin: namely, upregulation of genes in the PP2A branch of the TORC1 pathway and downregulation of genes in the *Sch9* branch of the TORC1 pathway (Figure 1B, top three rows). In contrast, glucose starvation, osmotic stress, heat stress, and oxidative stress all trigger downregulation of genes in the *Sch9* branch of the pathway, but have little influence on genes regulated by TORC1-PP2A (Figure 1B, middle four rows).

The condition-dependent expression patterns described above are especially clear when the rapamycin treatment and nitrogen starvation data are compared to the glucose starvation and osmotic stress data: TORC1-PP2A-dependent genes (Figure 1B, 101 genes) are induced an average of 6.2-fold in rapamycin and 7.7-fold in nitrogen starvation conditions, but only 1.5-fold in glucose starvation conditions and 1.2-fold in osmotic stress conditions (Table S2, cluster 2). Furthermore, while >70% of TORC1-PP2A-dependent genes are induced ≥ 3 -fold in rapamycin and nitrogen starvation conditions, only 12% and 3% of TORC1-PP2A-dependent genes are induced ≥ 3 -fold in glucose starvation and osmotic stress conditions (Figure 1C). Yet, all four stress/starvation conditions trigger a similar level of TORC1-Sch9-dependent gene repression (3.1- to 3.9-fold average repres-

sion; Figure 1B, 450 genes), including strong (>2.5-fold) repression of at least 156 of the 180 genes repressed ≥ 4 -fold in nitrogen starvation conditions (Table S2, cluster 4).

Oxidative stress and heat stress also fail to activate the TORC1-PP2A-dependent genes (1.4- and 1.5-fold average induction, respectively; Table S2 and Figure 1, B and C), but trigger less repression of the TORC1-Sch9-dependent genes (2.0- and 2.5-fold average repression, respectively; Figure 1B and Table S2) than glucose starvation, nitrogen starvation, and osmotic stress.

Together, these data show that the TORC1-PP2A-dependent genes, involved in amino acid synthesis and nitrogen assimilation, are activated to a significant level (>1.5-fold) only when cells are treated with rapamycin or starved for amino acids/nitrogen. In contrast, most stress and starvation conditions trigger robust (2.5- to 3.9-fold) downregulation of the TORC1-Sch9-dependent protein and ribosome synthesis genes.

Condition-dependent signaling through TORC1-Sch9

Previous studies have shown that TORC1 cooperates with several signaling pathways to control gene expression, including the cAMP-dependent protein kinase pathway (PKA pathway) (Broach 2012). The PKA kinases (*Tpk1/Tpk2/Tpk3*) are activated by glucose and act in parallel with TORC1-Sch9 to regulate *Dot6*, *Tod6*, *Sfp1* (Marion *et al.* 2004; Zurita-Martinez and Cardenas 2005; Lippman and Broach 2009; Zaman *et al.* 2009), and thus the ribosome and protein synthesis genes (Figure 1B, bottom row). Therefore, to determine whether the gene expression patterns we discovered in our initial microarray experiments match the output of the TORC1 pathway, or are driven by other signaling pathways such as PKA, we followed the phosphorylation of proteins regulated by TORC1.

To start, we monitored phosphorylation of six TORC1 target sites in the C terminus of the S6 kinase, *Sch9*, using a bandshift assay (see *Materials and Methods*) (Urban *et al.* 2007). We found that these sites are rapidly and completely dephosphorylated in rapamycin and all stress/starvation conditions (Figure 2A). We also found that the transcriptional repressors *Dot6* and *Tod6* (Figure 1A), targets of *Sch9* (Huber *et al.* 2009, 2011), are rapidly dephosphorylated in all stress and starvation conditions (Figure 2A).

In contrast, when we exposed a strain carrying mutations that render all three PKA kinases sensitive to the inhibitor 1-NM-PP1 (*Tpk1/Tpk2/Tpk3^{as}*), to saturating amounts of 1-NM-PP1 (Bishop *et al.* 2000; Zaman *et al.* 2009), we saw no change in *Sch9* phosphorylation (Figure 2B).

Next, to determine whether stress and starvation also block signaling through other channels in the *Sch9* branch of the TORC1 pathway (Figure 1A), we followed the nuclear localization of *Sfp1*, *Dot6*, and *Tod6*, using fluorescence microscopy and YFP-tagged constructs (Figure 2C). We found that the transcriptional activator *Sfp1* moves to the cytoplasm (Jorgensen *et al.* 2004; Marion *et al.* 2004), and the transcriptional repressors *Dot6* and *Tod6* move to the

Figure 2 TORC1 signaling is condition dependent. (A) Bandshift assays following the phosphorylation of Sch9, Dot6, and Tod6 (see *Materials and Methods* for details). (B) Bandshift assays following the phosphorylation of Sch9 and Gln3 in both the presence (wt + 1NM-PP1) and the absence (Tpk1-3^{AS} + 1NM-PP1) of Tpk1-3 activity. (C) Fluorescence microscopy was used to follow the localization of Dot6-YFP, Tod6-YFP, and Sfp1-YFP in different stress and starvation conditions. The fraction of cells with a detectable nuclear signal (inset number) was calculated by visually inspecting the images of 75–200 cells. *Sfp1 remains in the nucleus of most cells in osmotic stress (72%), but also partially relocalizes to the cytoplasm, so that the nuclear concentration is 54% of that found in SD medium (based on the intensity of nuclear fluorescence, $P < 1e-40$ by a *t*-test). Relocalization of Sfp1, Dot6, and Tod6 has previously been shown to depend on TORC1 activity (Marion *et al.* 2004; Huber *et al.* 2009, 2011). (D) Bandshift assays following the phosphorylation of PP2A branch proteins Npr1, Nnk1, and Gln3. Repeat experiments for A and D are shown in Figure S3.

nucleus, in all stress and starvation conditions examined. In the case of Dot6-YFP and Tod6-YFP, 2–3% of cells had a detectable nuclear signal in complete medium, but this increased to between 48% and 97% of cells after exposure to stress/starvation conditions (Figure 2C). In the case of Sfp1, 88% of cells had a detectable nuclear signal in complete medium, but this number decreased to 0–8% of cells in stress/starvation conditions. The only exception was in osmotic stress, where Sfp1 remains in the nucleus of most cells (72%), but partially relocalizes to the cytoplasm, so that the average nuclear concentration is only 54% of that found in complete medium (Figure 2C).

Putting these data together, we conclude that TORC1-Sch9 signaling is inhibited in all stress and starvation conditions, as suggested by our microarray data. Inhibition of TORC1-Sch9 signaling could be due to direct repression

of TORC1 activity and/or the action of as yet unknown proteins that block TORC1 signaling to Sch9 and other downstream factors.

Condition-dependent signaling through the PP2A branch of the TORC1 pathway

To determine whether PP2A-branch signaling is condition dependent, we monitored the phosphorylation of three factors that are regulated by protein phosphatase 2A and display a clear bandshift in response to rapamycin (Npr1, Nnk1, and Gln3; Figure 2D, column 1; see *Materials and Methods*).

We found that Npr1, Nnk1, and Gln3 are all rapidly and completely dephosphorylated in nitrogen starvation conditions, as found in rapamycin (compare columns 1 and 2 in Figure 2D). In contrast, dephosphorylation of Npr1, Nnk1,

Figure 3 Gtr1/2, Npr2/3, and Rho1 regulate TORC1-Sch9 signaling. (A) Bandshift assays following Sch9 phosphorylation in *GTR1/2B*, *npr2/3Δ*, *rho1B*, and wild-type strains. (B) Quantitation of the bandshift data from A, showing the average and standard deviation from at least two replicates (see *Materials and Methods* for details). The data are normalized to set the wild-type values to 1 at $t = 0$ min and 0 at $t = 5$ min. *Strains with a statistically significant defect ($P < 0.05$ in a t -test) are marked with an asterisk and highlighted in red in A. Repeat experiments are shown in Figure S3.

and *Gln3* does not occur, or is limited, in other stress and starvation conditions: (1) During glucose starvation, *Npr1*, *Nnk1*, and *Gln3* remain phosphorylated or become more phosphorylated (compare columns 2 and 3 in Figure 2D); (2) in osmotic stress, *Npr1*, *Nnk1*, and *Gln3* all remain phosphorylated (compare columns 2 and 4 in Figure 2D); and (3) in oxidative and heat stress, *Npr1* and *Gln3* are dephosphorylated/partially dephosphorylated, while *Nnk1* remains phosphorylated (compare columns 2, 5, and 6 in Figure 2D).

Putting these data together, we conclude that PP2A-branch signaling is strong in rapamycin and nitrogen starvation conditions, low or off in glucose starvation and osmotic stress, and weak/moderate in oxidative and heat stress. These results fit well with our microarray data and suggest that strong PP2A-branch signaling is required to activate the PP2A-dependent gene expression program (Figure 1B). Low-level signaling through the PP2A branch of the TORC1 pathway, in glucose starvation and noxious stress, may be due to poor activation of *Tap42*/PP2A and/or the action of other (unknown) signaling pathways on *Npr1*, *Nnk1*, and *Gln3*.

Context-dependent signaling through the TORC1 pathway

Our discovery that PP2A-branch signaling is on in rapamycin and nitrogen starvation and off, or mostly off, in glucose starvation and osmotic stress led us to ask how the pathway responds to combinations of stimuli.

First, we explored the influence that glucose has on the response to nitrogen starvation. As discussed earlier, nitrogen starvation in high-glucose medium triggers robust activation

of PP2A-branch signaling (Figure 2D, column 2). However, simultaneous nitrogen plus glucose starvation fails to activate PP2A-branch signaling, as judged by bandshift assays (compare columns 2 and 7 in Figure 2D) and microarray analysis (Figure S2). This glucose-dependent gating does not appear to depend on the PKA pathway, as TORC1 signaling in nitrogen starvation conditions occurs normally in the absence of *Tpk1/Tpk2/Tpk3* activity (Figure 2B).

Next, we examined the influence that glucose has on the response to rapamycin. The results match those for glucose and nitrogen starvation: Rapamycin treatment in the presence of glucose triggers activation of the PP2A branch of the TORC1 pathway (Figure 2D, column 1), while simultaneous rapamycin and glucose starvation does not (compare columns 1 and 8 in Figure 2D and Figure S2).

Finally, we examined TORC1 signaling in osmotic stress plus nitrogen starvation. Simultaneous osmotic stress plus nitrogen starvation activates the PP2A branch of the TORC1 pathway almost as well as nitrogen starvation alone, as judged by bandshift assays (compare columns 2 and 9 in Figure 2D) and microarray analysis (Figure S2).

Together, these data show that nitrogen starvation and rapamycin activate PP2A-branch signaling to a high level only when the cell has adequate glucose/energy.

Condition-dependent regulation of the TORC1 pathway

***Npr2/3*, *Rho1*, and *Gtr1/2* signaling:** Previous studies of TORC1 regulation revealed that the Rag and Rho GTPases, both located on the vacuolar/lysosomal membrane, inactivate TORC1 signaling in poor growth conditions (Sancak *et al.* 2008, 2010; Binda *et al.* 2009; Bonfils *et al.* 2012;

Figure 4 Gtr1/2, Npr2/3, and Rho1 regulate PP2A-branch signaling. (A and B) Bandshift assays following Par32 phosphorylation in wild-type (A) and *GTR1/2B*, *npr2/3Δ*, and *rho1B* (B) strains. (C) Quantitation of the bandshift data from B, showing the average and standard deviation from at least two replicates. The data are normalized so that wild-type values are 0 at $t = 0$ min in all conditions, 1 at $t = 5$ min in nitrogen starvation conditions, and -1 at $t = 5$ min in glucose starvation conditions. Note that while the *GTR1/2B* and *npr2/3Δ* strains have lower basal phosphorylation of Par32, both strains have a wild-type-like response to rapamycin (Figure S4) and thus the TORC1-PP2A pathway is still functional in these strains; it is just not activated by nitrogen starvation. *Strains with a statistically significant defect in the response to a given stimuli ($P < 0.05$ in a t -test) are marked with an asterisk and highlighted in red in B. Repeat experiments are shown in Figure S4.

Yan *et al.* 2012). Specifically, the Rag proteins (*Gtr1* and *Gtr2* in yeast) bind and inactivate TORC1 when amino acid levels fall below a critical level (Bonfils *et al.* 2012; Han *et al.* 2012), while *Rho1* binds and inactivates TORC1 in nitrogen starvation (and other) conditions (Yan *et al.* 2012). Importantly, active *Rho1* binds TORC1 in a manner that alters the interaction between TORC1 and *Tap42*, leading to activation of the PP2A complex (Yan *et al.* 2012). *Npr2/3*, part of the SEA complex that transiently associates with the vacuole, also transmits nitrogen (glutamine) starvation signals to TORC1 (Neklesa and Davis 2009; Dokudovskaya *et al.* 2011). Therefore, it appears that *Gtr1/2*, *Rho1*, and *Npr2/3* work together to drive the TORC1 pathway into the previously identified nitrogen starvation (*Sch9* off, PP2A on) state. But do these same signaling proteins play a role in driving TORC1 into the stress and/or glucose starvation states?

To address this question we created three strains, each with a mutation or mutations that stop *Gtr1/Gtr2*, *Rho1*, or *Npr2/Npr3* from transmitting nutrient signals to TORC1. We (1) blocked *Gtr1/2* signaling using mutations that lock *Gtr1* and *Gtr2* in their activated states [*GTR1/2B* (Binda *et al.* 2009)], (2) blocked *Rho1* signaling by deleting the *Rho1* activator *Rom2* [*rho1B* (Yan *et al.* 2012)], and (3) blocked *Npr2/3* signaling by deleting *Npr2/3* [*npr2/3Δ* (Neklesa and Davis 2009)] and then measured TORC1-Sch9 signaling in the *GTR1/2B*, *rho1B*, and *npr2/3Δ* strains.

We found that *Gtr1/2*, *Rho1*, and *Npr2/3* are all required for TORC1-Sch9 signaling in nitrogen starvation conditions (38–50% *Sch9* phosphorylation remaining after 5min, compared to 0% in wild-type cells; Figure 3, A and B). *Gtr1/2* and *Rho1* may also play a small role in TORC1-Sch9 signal-

ing in glucose starvation conditions (Figure 3, A and B). However, *Npr2/3*, *Gtr1/2*, and *Rho1* do not appear to influence TORC1-Sch9 signaling in osmotic stress, oxidative stress, or heat stress (Figure 3, A and B).

Next, we sought to determine whether *Gtr1/2*, *Rho1*, and *Npr2/3* influence signaling through the PP2A branch of the TORC1 pathway. Unfortunately, we could not perform these experiments using the *Gln3*, *Npr1*, or *Nnk1* bandshift assays described earlier because the gel mobility shifts were too small to accurately quantify the partial phosphorylation defects found in the *GTR1/GTR2B*, *rho1B*, and *npr2/3Δ* strains (*Gln3* data shown in Figure S5). To circumvent this problem, we searched for proteins in the PP2A branch of the TORC1 pathway that display a large mobility shift after rapamycin treatment. This led us to the *Npr1* target *Par32* (Figure 1A) (Huber *et al.* 2009). We found that *Par32* is hyperphosphorylated in nitrogen starvation and rapamycin (presumably due to activation of the *Par32* kinase, *Npr1*) and dephosphorylated in glucose starvation (possibly due to inactivation of PP2A and *Npr1*) (Figure 4A).

In line with our results for *Sch9*, we found that *Gtr1/2* and *Npr2/3* are required for *Par32* phosphorylation (PP2A-branch activation) in nitrogen starvation conditions (Figure 4, B and C), but not *Par32* dephosphorylation (PP2A-branch repression) in glucose starvation conditions (Figure 4, B and C). *Rho1* may also have a small impact on *Par32* phosphorylation (PP2A-branch activation) in nitrogen starvation (note the residual dephosphorylated *Par32* in Figure 4B), but does not influence *Par32* phosphorylation (PP2A-branch inhibition) in glucose starvation conditions (Figure 4, B and C).

Putting our *Sch9* and *Par32* bandshift data together with previous data (Sancak *et al.* 2008, 2010; Binda *et al.* 2009;

Figure 5 Snf1 and Hog1 regulate TORC1 signaling. (A) Bandshift assays following Sch9 phosphorylation in *snf1Δ*, *hog1Δ*, and wild-type cells. (B) Quantitation of the bandshift data from A, showing the average and standard deviation from at least two replicates (see *Materials and Methods* for details). The data are normalized to set the wild-type values to 1 at $t = 0$ min and 0 at $t = 5$ min. *Strains with a statistically significant defect ($P < 0.05$ in a t -test) are marked with an asterisk and highlighted in red in A. (C) Bandshift assays following Par32 phosphorylation in *snf1Δ*, *hog1Δ*, and wild-type cells. (D) Quantitation of the bandshift data from C, showing the average and standard deviation from at least two replicates. The data are normalized so that wild-type values are 0 at $t = 0$ min in all conditions, 1 at $t = 5$ min in nitrogen starvation conditions, and -1 at $t = 5$ min in glucose starvation conditions. *Strains with a statistically significant defect in the response to a given stimulus ($P < 0.05$ in a t -test) are marked with an asterisk and highlighted in red in A. The results for stress experiments are the same when we compare bandshifts in SD and YEPD medium, except for in the *hog1Δ* strain, where the data show that Hog1 regulates the TORC1-Sch9 signaling only in YEPD medium (Figure S6). Repeat experiments are shown in Figure S3 and Figure S4.

Bonfils *et al.* 2012; Yan *et al.* 2012), we conclude that Npr2/3, Gtr1/2, and Rho1 act primarily as nitrogen/amino acid-dependent regulators of TORC1, driving the pathway into the Sch9 off, PP2A strong/on, state.

Snf1/AMPK and Hog1/p38: Next, we focused on identifying the signaling protein(s) that regulates the TORC1 pathway in stress and glucose starvation conditions. Previous studies in human cells have shown that AMPK (Snf1 in yeast) inactivates TORC1 by phosphorylating the key regulatory subunit Raptor/Kog1 (Gwinn *et al.* 2008). Since AMPK is activated when energy/glucose levels are low, and in some stress conditions (Hedbacker and Carlson 2008), we hypothesized that Snf1 drives the TORC1 pathway into the glucose starvation and/or stress states. To test

this idea we examined TORC1 pathway signaling in an *snf1Δ* strain.

We found that Snf1 is important for inactivation of TORC1-Sch9 signaling in glucose starvation conditions ($50 \pm 6\%$ Sch9 phosphorylation remains after 5min, compared to $0 \pm 20\%$ in wild-type cells; Figure 5, A and B). However, Snf1 does not influence TORC1-Sch9 signaling in nitrogen starvation or stress conditions ($<8\%$ Sch9 phosphorylation after 5 min; Figure 5, A and B). Snf1 is also required for Par32 dephosphorylation (PP2A-branch inhibition) in glucose starvation conditions (Figure 5, C and D), but does not affect Par32 phosphorylation (PP2A-branch activation) in nitrogen starvation conditions (Figure 5, C and D).

In line with our bandshift data, we also found that Snf1 is required for downregulation of the TORC1-Sch9-dependent

Figure 6 Gene regulation by the Snf1 and Hog1 kinases. (A) Snf1 regulates the TORC1-dependent gene expression program in glucose starvation conditions. The global gene expression program activated in nitrogen starvation, 0.4 M KCl stress, and glucose starvation was measured in both *snf1Δ* and wild-type cells by comparing the mRNA levels before (Cy3, green) and after 20 min of stress/starvation (Cy5, red), on a two-color DNA microarray (red/green columns). These data were also used to calculate the difference between the transcription programs activated in *snf1Δ* and wild-type cells by subtracting the $\log_2$ expression values in the *snf1Δ* strain from those in the wild-type strain (blue/yellow columns). (B) Hog1 regulates the TORC1-dependent gene expression program in salt stress conditions. The gene expression program activated in 0.4 M KCl was measured in both *hog1Δ* and wild-type cells (in YEPD medium), and the expression difference between the two strains was calculated, as described above, for the *snf1Δ* cells.

genes in glucose starvation conditions, but has little to no impact on the expression of TORC1-dependent genes in nitrogen starvation and osmotic stress conditions (Figure 6A).

Putting these data together, we conclude AMPK/Snf1 plays a key role in driving the TORC1 pathway into the glucose starvation state (Sch9 off, PP2A low/off). However, *snf1Δ* cells still do not activate the PP2A pathway in glucose plus nitrogen starvation conditions (Figure S4 and Figure S5), suggesting that at least one additional (unknown) factor cooperates with Snf1 to block signaling through the PP2A branch of the TORC1 pathway.

Finally, to determine which proteins inactivate the TORC1 pathway during stress, we examined signaling in several strains (*hyr1Δ*, *skn7Δ*, *rad9Δ*, *ybp1Δ*, and *hog1Δ* cells), each missing one stress-activated protein. This revealed that the MAPK Hog1/p38 acts to inhibit TORC1-Sch9 signaling in osmotic stress ($39 \pm 1\%$ Sch9 phosphorylation remains after 5 min, compared to $0 \pm 2\%$ in wild-type cells; Figure 5, A and B). Consistent with this result, deletion of *HOG1* causes a significant defect in the repression of TORC1-Sch9-dependent genes in osmotic stress conditions (1.9-fold average defect among the top 100 repressed genes in the TORC1-Sch9 group; Figure 6B and Table S2). However, *Hog1* does not inhibit TORC1-Sch9 signaling in other stress and starvation conditions (Figure 5, A and B) or during

osmotic stress in minimal (SD) medium (Figure S6), indicating that additional (unknown) signaling pathways regulate TORC1 and/or TORC1 pathway activity in osmotic, oxidative, and heat stress.

Discussion

Early studies of TORC1 signaling focused on examining the response to rapamycin, nitrogen starvation, and amino acid starvation. All three stimuli were found to lead to the same change—inhibition of TORC1-Sch9 signaling and activation of TORC1-PP2A signaling. It therefore appeared as though all stress/starvation signals affect the TORC1 pathway in the same way. This simple view of TORC1 pathway signaling began to break down when the Hall and Cooper laboratories showed that different types of nitrogen starvation lead to different responses through the PP2A branch of the pathway (Crespo *et al.* 2002; Georis *et al.* 2011; Rai *et al.* 2013; Tate and Cooper 2013). Now, in this work, we show that glucose starvation, osmotic stress, oxidative stress, and heat stress all drive the TORC1 pathway into a completely distinct set of states, in which Sch9-branch signaling and PP2A-branch signaling are both inhibited. Furthermore, we show that nitrogen starvation and rapamycin activate PP2A-branch signaling only when the cell has adequate glucose/energy.

Figure 7 Model of the TORC1 signaling circuit in yeast. The TORC1 circuit behaves as an energy management system with specific nutrient, energy, and stress sensors driving the system between alternate signaling states, shown schematically in red and green boxes. See the text for details.

Our observation that nitrogen/amino acid starvation leads to robust PP2A-branch signaling, while other stress/starvation conditions lead to weak or no PP2A-branch signaling, seems to contradict previous reports indicating that PP2A is active in stress (Crespo *et al.* 2001; Santhanam *et al.* 2004; Yan *et al.* 2012). However, there are technical reasons for the discrepancy. The most important factor is that previous studies did not examine PP2A signaling in osmotic stress or glucose starvation—the two conditions where we see little to no PP2A-branch activity. The other factor is that we examined PP2A-branch signaling using multiple probes, while previous studies monitored the phosphorylation of only one protein or two highly related proteins (*e.g.*, Gat1 and/or Gln3). In line with previous results we find that Gln3 is dephosphorylated in oxidative and heat stress, but we nevertheless conclude that PP2A-branch signaling is weak in these conditions based on phosphorylation data for Nnk1 and Npr1 and microarray

data. Thus, our results are consistent with published data but offer a broader view of PP2A-branch signaling and activity.

On top of building a map of input:output characteristics of the TORC1 pathway, the data presented in this study also reveal that different signaling pathways drive the TORC1 pathway into each of its signaling states.

First, we show that the known TORC1 regulators Gtr1/2, Npr2/3, and Rho1 have a strong impact on TORC1 signaling in nitrogen/amino acid starvation conditions, but not glucose starvation or stress. These data fit well with previous observations for Npr2/3 and Gtr1/2 and data showing that Gtr1/2 are regulated in response to glutamine and leucine signals (Sancak *et al.* 2008; Binda *et al.* 2009; Neklesa and Davis 2009; Bonfils *et al.* 2012; Duran *et al.* 2012; Han *et al.* 2012; Panchaud *et al.* 2013). Our data also fit with a recent study that showed that Rho1 inhibits TORC1 signaling in nitrogen starvation conditions (Yan *et al.* 2012).

The relationship between our data for *Rho1* and the data of Yan *et al.* (2012) deserves additional comment. Yan *et al.* (2012) conclude that *Rho1* plays a key role in transmitting stress signals to TORC1. Yet, there is no overlap between the conditions examined in Yan *et al.* (2012) and our study (outside of the nitrogen starvation data discussed above). They show that *Rho1* activation is required for TORC1 inactivation in calcofluor white, caffeine, and rapamycin treatment, while we show that *Rho1* activation has no impact on TORC1 signaling in osmotic stress, oxidative stress, or heat stress. Putting the two sets of data together, we suggest that *Rho1* plays a role in regulating TORC1 during cell wall damage (as induced by calcofluor white), consistent with the known connection between the cell wall integrity (PKC) pathway and *Rho1* (Levin 2011). *Rho1* also seems to be required for the action of the direct TORC1 inhibitors, caffeine and rapamycin. However, *Rho1* does not appear to be involved in transmitting oxidative, osmotic, and heat stress signals to TORC1.

Second, we show that the AMP-activated protein kinase (AMPK), *Snf1*, represses TORC1-Sch9 signaling and hyper-inactivates PP2A-branch signaling in glucose starvation, but not other conditions. We also show that *Snf1* is required for repression of the TORC1-dependent gene expression program in glucose starvation conditions. These results show that AMPK regulates the TORC1 pathway (and possibly TORC1 itself) in yeast, as found in mammalian cells (Inoki *et al.* 2003; Gwinn *et al.* 2008).

It is worth noting that our data for *Snf1* stand in conflict with conclusions drawn in a previous study of TORC1-*Snf1* signaling by Zhang *et al.* (2011). Zhang *et al.* (2011) compared the global transcription and phosphorylation changes that occur in *tor1Δ* and *snf1Δ* cells in several stress and starvation conditions, including glucose starvation. They then argued that TORC1 and *Snf1* must act independently since deletion of *TOR1* has significantly less influence on transcription and protein phosphorylation than deletion of *SNF1*. We submit that this logic is flawed since budding yeast have two TOR genes, and deletion of *TOR1* has little impact on TORC1 signaling, due to the compensatory activity of *Tor2* (Helliwell *et al.* 1994; Loewith *et al.* 2002). In fact, we observe that the potent TORC1 inhibitor rapamycin causes expression changes similar to those found in complete glucose starvation (Figure 1B) and deletion of *Snf1* (Figure 6A), consistent with our data showing that *Snf1* regulates TORC-Sch9 and PP2A-branch signaling.

Finally, we show that the MAPK *Hog1*/p38 is required for inhibition of TORC1-Sch9 signaling in osmotic stress conditions (in rich medium), but not other stress/starvation conditions. We believe this is the first time *Hog1* has been linked to TORC1 pathway regulation.

Putting all of our data together, we are able to construct an expanded model of the TORC1 pathway (Figure 7). This model highlights, for the first time, the way that cells process and integrate information from distinct stress and starvation stimuli: When glucose/energy levels fall, AMPK and

at least one additional regulator drive the TORC1 pathway into an off state (red panel in Figure 7). By contrast, in the presence of glucose/energy, the TORC1 pathway can take up at least three distinct states (green panels in Figure 7). First, in nutrient rich and stress free conditions, TORC1 phosphorylates proteins in the *Sch9* branch of the pathway to promote protein synthesis/growth. However, if the cell begins to run out of amino acids or nitrogen, the *Gtr1/2* and *Rho1* GTPases, working together with *Npr2/3*, bind TORC1 to inhibit signaling through the *Sch9* branch of the pathway and activate signaling through Tap42-PP2A. This diverts energy away from protein synthesis and toward the production of key metabolites such as amino acids. In other words, AMPK tells the TORC1 pathway how much energy the cell has to spend, while nutrient signals through *Gtr1/2* and *Rho1* tell the TORC1 pathway whether the cell should spend this energy producing missing metabolites or driving mass accumulation. Finally, if cells are exposed to stress, *Hog1*/p38 and other unknown factors transiently inhibit TORC1-Sch9 while cellular resources are used to promote a TORC1-independent stress response.

While our model provides important insight into cell function and the overall design of the TORC1 pathway, it is far from complete. In particular, it remains unclear how glucose starvation and stress signals regulate the TORC1 pathway. One possibility is that stress and glucose starvation signals act directly on TORC1 to inhibit PP2A activation and/or TOR kinase activity. However, glucose/stress signals may also act on other components in the TORC1 pathway, including *Sch9*; Tap42/PP2A; and/or *Npr1*, *Nnk1*, and *Gln3*. Distinguishing between these and other possible scenarios will have to await mechanistic studies of *Snf1*, *Hog1*, and TORC1 pathway signaling in glucose starvation and stress conditions.

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GENETICS

Supporting Information

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State Transitions in the TORC1 Signaling Pathway and Information Processing in *Saccharomyces cerevisiae*

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SUPPLEMENT

Supplemental Figures

Figure S1 The gene expression program activated by the TORC1 pathway depends on the cellular condition. The global gene expression program activated in a range of stress and starvation conditions was measured by comparing the mRNA levels before (Cy3, green) and after 20 min of stress/starvation (Cy5, red), on a two-color DNA microarray (as described in the Methods). We then filtered this data, using the rapamycin response, to identify the genes regulated by TORC1 (at a 2-fold cut-off). Clustering the data for the 1174 TORC1 dependent genes led to the identification of six distinct gene modules (Table S2). The three major gene modules are; (Group II) 101 genes involved in nitrogen and amino acid metabolism, known targets of TORC1 through PP2A; (Group III) 426 Stress and carbohydrate metabolism genes; and (Group IV) 450 Ribosome and protein synthesis genes, targets of TORC1 through Sch9. The genes in Group III were identified as targets of the general stress transcription factors Msn2 and Msn4 by comparing our expression data to a previously published list of Msn2/4 gene targets (CAPALDI *et al.* 2008), shown here as a column labeled Msn2/4 dependent (black boxes, Column I). Since Msn2/4 are primarily regulated by factors other than TORC1, such as PKA, Hog1 and Snf1 (GÖRNER *et al.* 1998; DE WEVER *et al.* 2005; CAPALDI *et al.* 2008; PETRENKO *et al.* 2013), we did not investigate this gene group further. Three minor gene modules were also identified. The first of these (Group I) is involved in amino-acid metabolism, and is activated in nitrogen and amino-acid starvation conditions, but not during glucose starvation or stress, just like Group II. However, the genes in Group I show limited (and mixed) dependence on rapamycin, so were not analyzed further here. The other two groups (Groups V and VI) show weak and mixed repression across conditions (including rapamycin), and thus were also dropped from our analysis. Finally, for comparison we also measured the gene expression changes that occur when all three PKA kinases (Tpk1-3) are inactivated for 20 min using the chemical inhibitor 1-NM-PP1 (Tpk1-3^{AS} + 1NM-PP1). Note that this experiment was carried out in a strain carrying point mutations in Tpk1-3 (Tpk1-3^{AS}) that render them inactive in the concentration of 1-NM-PP1 used here (100nM, (ZAMAN *et al.* 2009)). The 1-NM-PP1 and Msn2/4 data were not included during clustering. Shown to the right of each cluster group are the major associated GO terms, as calculated using GO Stat. A complete list of GO terms and the transcription factor binding sites over-represented in each gene group (along with gene descriptions and expression data) can be found in Table S2. The microarray data shown in this figure, and throughout the paper, are available in Table S1.

Figure S2 PP2A dependent gene expression in combined stress conditions. Gene expression profiling by microarray reveals that TORC1/PP2A dependent reprogramming of metabolism occurs in nitrogen starvation conditions, but not under conditions of noxious environmental stress or glucose deprivation. Moreover, while the addition of rapamycin, or the removal of nitrogen, are sufficient to trigger metabolic reprogramming in glucose rich environments, neither stimuli is potent enough to overcome the barrier to this transition imposed by the removal of glucose. Specifically, PP2A branch pathway genes are induced 6.2 and 7.7-fold on average in rapamycin and N₂ starvation; 1.5-fold average induction in glucose starvation, 1.2-fold, 1.5-fold, and 1.5-fold in KCl, heatshock, and oxidative stress exposure; and 1.7-fold on average in glucose starvation with rapamycin and/or nitrogen starvation. The genes shown here are Group II in Figure S1.

Figure S3 Sch9 mobility shift dataset. Sch9 phosphorylation was measured using SDS PAGE and an anti-HA western blot as described for Figs. 2, 3 and 5.

Figure S4 Par32 mobility shift dataset. Par32 phosphorylation was measured using SDS PAGE and an anti-Myc western blot as described for Figs. 4 and 5.

Figure S5 Gln3 mobility shift dataset. Gln3 phosphorylation was measured using SDS PAGE and an anti-Myc western blot as described for Fig. 2. A horizontal line through the approximate midpoint of Gln3 phosphorylation at t=0 has been overlaid on each image to assist in determining Gln3 mobility shifts in response to environmental conditions.

Figure S6 TORC1 Regulation in SD medium. Control experiments for Figs. 3 and 5 indicating Gtr1/2, Npr2/3, Rho1, Snf1, and Hog1 do not regulate TORC1 in osmotic stress, oxidative stress, or heat stress in SD. Experiments and analysis were carried out as described for Figs. 3a and b.

Supplemental Tables

Table S1 Complete microarray data (tab 1) and the TORC1 dependent gene list (tab 2). Table S1 is available as an Excel (.xlsx) file at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.168369/-/DC1>.

Table S2 Gene descriptions, expression data, and Ontology Assignments and Transcription Factor Analysis for Gene Modules in Fig. S1. Gene Ontology terms were identified using GO Stat (BEISSBARTH AND SPEED 2004), and all over-represented terms with a statistical cut-off of $p < 1.0 \times 10^{-5}$ are shown here. Group V had no significant GO terms, even at a cutoff of $p < 0.001$. The transcription factors regulating each module were identified using YEASTRACT (TEIXEIRA *et al.* 2006; MONTEIRO *et al.* 2008; ABDULREHMAN *et al.* 2011; TEIXEIRA *et al.* 2014), examining direct binding data, with a cutoff of $p < 0.001$. Note that each gene module (from Fig. S1) has its own tab within the spreadsheet. Table S2 is available as an Excel (.xlsx) file at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.168369/-/DC1>

Table S3 *Saccharomyces cerevisiae* strains used in this study. Table S3 is available as an Excel (.xlsx) file at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.168369/-/DC1>

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