Indirect monitoring of TORC1 signalling pathway reveals molecular diversity among different yeast strains

Running title: Diversity among yeast strains in TORC1 pathway activation

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ABSTRACT

Saccharomyces cerevisiae is the main species responsible for the alcoholic fermentation in wine production. One of the main problems in this process is the deficiency of nitrogen sources in the grape must, which can lead to stuck or sluggish fermentations. Currently, yeast nitrogen consumption and metabolism are under active inquiry, with emphasis on the study of the TORC1 signalling pathway, given its central role responding to nitrogen availability and influencing growth and cell metabolism. However, the mechanism by which different nitrogen sources activates TORC1 is not completely understood. Existing methods to evaluate TORC1 activation by nitrogen sources are time-consuming, making difficult the analyses of large numbers of strains. In this work, a new indirect method for monitoring TORC1 pathway was developed based on the luciferase reporter gene controlled by the promoter region of *RPL26A* gene, a gene known to be expressed upon TORC1 activation. The method was tested in strains representative of the clean lineages described so far in *S. cerevisiae*. The activation of the TORC1 pathway by a proline-to-glutamine upshift was indirectly evaluated using our system and the traditional direct methods based on immunoblot (Sch9 and Rps6 phosphorylation). Regardless the different molecular readouts obtained with both methodologies, the general results showed a wide phenotypic variation between the representative strains analysed. Altogether, this easy-to-use assay opens the possibility to study the molecular basis for the differential TORC1 pathway activation, allowing to interrogate a larger number of strains in the context of nitrogen metabolism phenotypic differences.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a species of industrial importance given its role in the production of bread and various alcoholic beverages, being the main species responsible for the alcoholic fermentation in the process that involves the transformation of grape must into wine (Pretorius, 2000). One of the main problems in the wine industry is the deficiency of nitrogen sources in the grape must, which are the key factors regulating the biomass content during the fermentation process and directly impacting the fermentation rate (Varela, Pizarro, & Agosin, 2004). Thus, nitrogen deficiencies can lead to stuck or sluggish fermentations, reducing the fermentation rate and generating economic losses for the industry (Taillandier, Ramon Portugal, Fuster, & Strehaiano, 2007).

Recently, the TORC1 signalling pathway activation during the fermentation process has gotten renewed attention due to its central role in nitrogen metabolism regulation (Tesniere, Brice, & Blondin, 2015). TOR kinases are key components of this eukaryotic signalling pathway that connects nutrient sufficiency to growth, promoting anabolic processes such as protein synthesis and ribosome biogenesis. There are two kinases (Tor1 and Tor2) in *S. cerevisiae* that are part of two protein complexes (TORC1 and TORC2), of which TORC1 is inhibited by rapamycin (Loewith & Hall, 2011; Loewith et al., 2002). Nutrients, especially nitrogen sources, activate TORC1 which lead to two main effectors of this pathway: the Sch9 kinase and the Tap42-PP2A phosphatase complex (Broach, 2012; Loewith & Hall, 2011).

Although proximal and distal effectors of TORC1 are well characterized, the mechanism by which nitrogen sources activate TORC1 is not completely understood (Conrad et al., 2014; Gonzalez & Hall, 2017). In this regard, it has been determined an amino acid-dependent mechanism of TORC1 activation through the EGO complex (EGOC), whose main components are the GTPases Gtr1 and Gtr2 (Hatakeyama & De Virgilio, 2016; Powis & De Virgilio, 2016).

However, it is unknown how amino acids are sensed, with the exception of leucine, which is thought to be sensed by the leucil-tRNA synthetase (Bonfils et al., 2012). The EGOC-dependent activation of TORC1 occurs rapidly but transiently by both poor and preferred nitrogen sources. In general, glutamine, glutamate, asparagine and ammonium sustains high specific growth rate in yeast, and are considered as preferred nitrogen sources. Conversely, proline, allantoin and urea allows slow growth rate in yeast, and are considered poor or non-preferred nitrogen sources (Crepin, Nidelet, Sanchez, Dequin, & Camarasa, 2012). Only preferred sources promote sustained activation coupled with an accumulation of intracellular glutamine, but independently of EGOC (Stracka, Jozefczuk, Rudroff, Sauer, & Hall, 2014). Furthermore, constitutive activity of EGOC fails to supress the TORC1 signalling defect under ammonium deprivation (Binda et al., 2009). Thus, there is an alternative mechanism of TORC1 activation independent of EGOC in which the participating proteins have not been fully determined (Chantranupong, Wolfson, & Sabatini, 2015; Gonzalez & Hall, 2017), with only suggested actors like Pib2 protein (Kim & Cunningham, 2015; Michel et al., 2017; Tanigawa & Maeda, 2017; Ukai et al., 2018; Varlakhanova, Mihalevic, Bernstein, & Ford, 2017).

New methodologies to study the activation of the TORC1 pathway in response to nitrogen sources have been developed, such as the recently developed *in vitro* TORC1 assay based on phosphorylation of 4EBP1, a well-known target of mTORC1 (mammalian TORC1) (Tanigawa & Maeda, 2017). Similarly, two methods based on immunoblot detection of TORC1 targets have proved a direct survey of its activity. The first of them uses the phosphorylation of the Sch9 kinase as readout, the best-characterized TORC1 direct target in yeast (Stracka et al., 2014), while the second uses the phosphorylation of the target ribosomal protein S6 (Rps6) (Gonzalez et al., 2015; Yerlikaya et al., 2016). These methods have allowed the phenotyping of TORC1 pathway activation in response to different amino acids. However, current methods are laborious, making difficult the analysis of larger number of yeast strains.

In this scenario, an alternative to study the TORC1 pathway is the use of genetic approaches, such as those that have been used to shed light into the molecular bases that underlie the phenotypic variability in nitrogen consumption in yeasts (Brice, Sanchez, Bigey, Legras, & Blondin, 2014; Contreras et al., 2012; Cubillos et al., 2017; Gutierrez, Beltran, Warringer, & Guillamon, 2013; Ibstedt et al., 2015; Jara et al., 2014). However, linkage approaches require phenotyping of a larger number of strains, which is unaffordable for monitoring TORC1 activity using the abovementioned methodologies based on immunoblot detection.

In this work, a new microculture-based methodology was developed to indirectly evaluate TORC1 activation in a nitrogen upshift experiment. Our approach utilizes the luciferase reporter gene controlled by the promoter region of *RPL26A* gene, a gene known to be expressed upon TORC1 activation, resulting in an indirect measuring of TORC1 activation. We used our method to indirectly evaluate TORC1 activity in four yeast strains belonging to the main phylogenetic lineages described so far, showing the existence of natural variation in TORC1 signalling pathway activation in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains and plasmids

The strains used in this work correspond to stable haploid versions of strains representative of four clean lineages previously described for *S. cerevisiae* (Liti et al., 2009). These strains are YPS128 (North American, 'NA'), Y12 (Sake, 'SA'), DBVPG6044 (West African, 'WA') and DBVPG6765 (Wine/European, 'WE') (Cubillos, Louis, & Liti, 2009). Strains were transformed using lithium acetate method (Gietz & Schiestl, 2007) with the pJU733 plasmid, which has the *SCH9*-3xHA insert and *URA3* gene as selectable marker (Urban et al., 2007).

The pRS426 plasmid carrying the firefly luciferase reporter gene and *URA3* gene as selection marker (*Luc-URA3* construct) was previously described (Salinas et al., 2016). This firefly luciferase reporter gene is a destabilized version that allows real-time quantification of gene expression *in vivo* (Rienzo, Pascual-Ahuir, & Proft, 2012). The construct *Luc-URA3* was amplified by PCR and used to replace the endogenous *RPL26A* ORF. Additionally, strains carrying *GTR1* deletion were generated replacing the ORF by the hygromycin cassette. All strains used are listed in Table 1.

Selection of *RPL26A*

A revision of previously described genes responding to TORC1 pathway was carried out to select candidate genes, considering those genes whose expression is strongly activated by the TORC1 signalling pathway. From the analysis of the TORC1-dependent transcriptome (Oliveira et al., 2015), we selected the ribosomal protein encoding gene *RPL26A*, due to its minor effects on translation and low pleiotropic effects generated by its deletion in a laboratory genetic background, according to the SGD (*Saccharomyces* Genome Database, www.yeastgenome.org).

Indirect monitoring of the TORC1 pathway activation in microculture conditions

The activation of the TORC1 pathway in the strains carrying the *Luc-URA3* reporter construct under the control of the *RPL26A* promoter (P_{RPL26A}) was evaluated by monitoring the optical density at 600 nm (OD_{600}) and luminescence (Lum) of the cells in microculture conditions (Salinas et al., 2016). We used a nitrogen (proline-to-glutamine or proline-to-leucine) upshift experiment, where the strains were grown until OD_{600} ~0.8 at 30 ºC in 96-well plates containing 300 µL of yeast minimal medium (1.7 g/L yeast nitrogen base without amino acids and without ammonium sulphate and 20 g/L glucose) with proline (0.5 mg/mL) as the only nitrogen source (YMM+Pro), supplemented with luciferin (1 mM) (Figure 1). Then, 10 µL of glutamine or

leucine (15 mg/mL; 0.5 mg/mL final concentration) were added. Luminescence was measured up to 12 hours using 5 min intervals in a Cytation 3 microplate reader (Biotek, USA). To check that luciferin is not limiting in the production of luminescence, we also added luciferin (25 mM; 1 mM final concentration) together with the glutamine pulse (Supplementary Figure 3). All the micro-cultivation experiments were carried out in three independent biological replicas.

Analysis of growth curves

Relative fitness variables (growth parameters) for each strain were calculated as previously described (Kessi-Perez et al., 2016; Warringer et al., 2011). Briefly, efficiency of proliferation (population density change), rate of proliferation (population doubling time) and lag of proliferation were extracted from high-density growth curves using Gompertz growth equation (Yin, Goudriaan, Lantinga, Vos, & Spiertz, 2003). Statistical analysis of these parameters consisted in Welch two sample t-tests, which were performed using R software (R-Core-Team., 2013).

Evaluation of the activation of TORC1 pathway by immunoblot

The activation of the TORC1 pathway in the strains carrying the pJU733 plasmid was evaluated by assessing both the phosphorylation of its proximal effector Sch9 and the ribosomal protein Rps6 in a nitrogen (proline-to-glutamine) upshift experiment (Gonzalez et al., 2015; Stracka et al., 2014). Briefly, strains were grown in flasks containing 50 mL of YMM+Pro medium until OD_{600} ~0.8 and then 700 µL of glutamine (25 mg/ml; 0.5 mg/mL final concentration) were added. Samples were taken at different time points (0, 5, 15 and 30 min) to perform protein extraction and subsequent immunoblot as previously described (Gonzalez et al., 2015). To evaluate Sch9 phosphorylation, cell extracts were subjected to chemical cleavage with 2-nitro-5-thiocyanatobenzoic acid (NTCB, Sigma). Antibodies used included anti-HA (Cell Signaling Technology Cat# 2367S, RRID:AB_10691311), phospho-Ser235/Ser236-S6 (Cell Signaling

Technology Cat# 2211, RRID:AB_331679), RPS6 (Abcam Cat# ab40820, RRID:AB_945319), peroxidase-Monoclonal Mouse Anti-Rabbit IgG (Jackson ImmunoResearch Labs Cat# 211-032-171, RRID:AB_2339149) and peroxidase-Goat Anti-Mouse IgG (Jackson ImmunoResearch Labs Cat# 115-035-174, RRID:AB_2338512).

RESULTS AND DISCUSSION

A new indirect method for monitoring TORC1 activation

We developed a new method to evaluate TORC1 activation by monitoring the luminescence produced by yeast cells in microculture conditions. We used the method to indirectly characterize TORC1 activity in four strains representatives of clean lineages previously described in *S. cerevisiae* (Liti et al., 2009). We chose the strains YPS128 (North American, NA), Y12 (Sake, SA), DBVPG6044 (West African, WA) and DBVPG6765 (Wine European, WE). These four strains were transformed with the reporter construct (*Luc-URA3*), designed to replace the endogenous ORF of *RPL26A*, such that luciferase expression is now under the control of the endogenous *RPL26A* promoter (P_{RPL26A}). We selected the *RPL26A* promoter as readout of our system since *RPL26A gene* showed strong expression upon TORC1 activation (Supplementary Figure 1, adapted from (Oliveira et al., 2015)). The growth and luciferase expression of the strains were evaluated in YMM+Pro medium, monitoring both the OD_{600} and luminescence of the cultures over time (Figure 1). Once cells reached OD_{600} ~0.8, a pulse of glutamine was added to continue recording luciferase expression until 12 hours (Figure 1). It is important to remark that we used strains auxotroph for uracil (Cubillos et al., 2009), being the unique auxotrophy present in those strains, and therefore, the reporter construction have *URA3* as selectable marker, avoiding the use of uracil in the YMM medium, which can be used as an alternative nitrogen source.

Initially, we evaluated the growth and luciferase expression under the control of P_{RPL26A} in the four strains expressing the reporter gene in YMM+Pro medium, showing a maximum level of luciferase expression at the beginning of the exponential phase and before OD_{600} ~0.8 (Figure 2). This behaviour is commonly observed for yeast promoters, with activity during a transient period at the exponential growth phase (Dolz-Edo, Rienzo, Poveda-Huertes, Pascual-Ahuir, & Proft, 2013; Rienzo et al., 2012; Rienzo et al., 2015). Interestingly, the WA strain showed a slower growth in comparison with the other three strains in this medium, reaching OD_{600} ~0,8 at later time points (Figure 2). Considering that OD_{600} ~0.8 is a requisite to perform the nitrogen upshift experiment, we overcome this problem by increasing ten-fold the concentration of WA strain at the beginning of the experiment, allowing us to evaluate the four strains in a single experiment. Additionally, we compared the growth parameters (lag time, rate and efficiency) for the strains carrying *RPL26A* deletion by luciferase (*rpl26a*Δ) versus the wild type (WT) strains. We observed a small growth defect in the *rpl26a*Δ strains compared to the WT strains when growth in YMM+Pro, with statistically differences in lag time and growth rate for the NA genetic background (Figure 2 and Table 2). Overall, the results showed that *RPL26A* promoter is active at the beginning of the exponential phase and before OD_{600} ~0.8, avoiding interferences with the signal produced by a nitrogen pulse at this OD. Additionally, *RPL26A* deletion generated minor effects over the growth parameters under the culture conditions assayed, in all four genetic backgrounds evaluated.

We then performed an upshift nitrogen experiment in a microplate reader for the four strains simultaneously, adding glutamine at OD_{600} ~0.8 and monitoring the luminescence of cells during 12 hours after glutamine addition. In all strains, the luciferase expression increased rapidly and then decreased to background levels 10-12 hours after the nitrogen pulse (Figure 3A). The NA and WA strains showed the more similar behaviour, with a similar first maximum of luciferase expression at 2-3 hours after nitrogen pulse and a second maximum at 6-8 hours

(Figure 3 and Table 3). The WE strain showed the smaller first maximum of luciferase expression but with a comparable second maximum with NA and WA strains, while SA strain showed the biggest first maximum and no second expression peak (Figure 3 and Table 3).

We evaluated the robustness of our method using strains carrying different mating types. We observed similar results when cells were subjected to a nitrogen upshift experiment (Supplementary Figure 2). Moreover, we demonstrated that luciferin depletion over time does not affect the results obtained, which was corroborated by performing a simultaneous addition of glutamine and luciferin in the nitrogen upshift experiment (Supplementary Figure 3). Finally, we assessed the capacity of our method to be used in haploid and diploid strains. For this, we generated a hybrid strain using the phenotypically more different strains (SA and WE strains), observing in the hybrid an intermediate phenotype with respect to the parent strains in nitrogen upshift experiments (Supplementary Figure 4).

In conclusion, using our method the SA and WE strains showed the more dissimilar phenotypes for luciferase expression after a nitrogen (proline-to-glutamine) upshift experiment, which can be consider an indirect measure of the TORC1 activity. Previously, these strains were also different for other phenotypes such as fermentation kinetics and fungicide resistance (Kessi-Perez et al., 2016), nitrogen consumption (Jara et al., 2014) and oenological traits (Salinas et al., 2012). The type of experiment performed in those studies and others, such as bulked segregant analysis (BSA) or comparative genomics, requires larger number of strains to be evaluated (Mackay, Stone, & Ayroles, 2009). Thus, the new method here developed could be an important tool to continue unravelling genetic determinants involved in the nitrogen sensing associated with the activation of the TORC1 signalling pathway.

Confirming phenotypic variability in TORC1 pathway activation between yeast strains

To corroborate the phenotypic diversity seen by our method, we evaluated Sch9 phosphorylation by Western blot in a proline-to-glutamine upshift experiment as readout of TORC1 activation (Figure 4). In general, phosphorylation of Sch9 increased after 5 min of glutamine addition, decreased after 15 min and raised up again at 30 min, consistent with results previously described (Stracka et al., 2014). This behaviour was observed in NA, SA and WA strains, with the SA strain showing the greater activation at 30 minutes (Figure 4A and 4B). However, the WE strain appears to lack a reactivation at 30 minutes, even though it shows a great activation 5 minutes after the glutamine pulse (Figure 4A and 4B). In general, these results are consistent with previously described observation where glutamine can activate TORC1 both dependent and independent of EGOC, with the activation at 5 minutes being EGOC-dependent and the activation at 30 minutes being EGOC-independent (Stracka et al., 2014). Interestingly, although the WA strain showed the expected behaviour for Sch9 phosphorylation (Figure 4A and 4B), the unphosphorylated isoform of Sch9 was highly abundant in this strain, confirming the variability in TORC1 activity for the analysed strains.

Using the same experimental strategy, we evaluated TORC1 activation by monitoring Rps6 phosphorylation. The NA and WA strains showed similar phenotypes, increasing Rps6 phosphorylation after the glutamine pulse (Figure 4A and 4C). Nevertheless, SA strain seems to have a greater activation, while WE strain shows both a lower activation and a decay at 30 min, in concordance with the results obtained for Sch9 phosphorylation (Figure 4). Overall, with exception of the WE strain, all strains showed the expected behaviour, in agreement with previously described observations where Rps6 phosphorylation increased over time in a nitrogen upshift experiment (Gonzalez et al., 2015).

Altogether, the results obtained for Sch9 and Rps6 phosphorylation confirmed the existence of phenotypic differences between strains, showing SA strain as the one that have a greater TORC1 activation and the WE strain as the one with the less sustained one. This general

conclusion agrees with the results obtained by our method, although a direct comparison between them is not possible. Nevertheless, we compared these results with the obtained using the microculture method, focusing in the first 4 hours after the nitrogen pulse, where the first luciferase expression maximum appears (Figure 3B and Table 3). When we compared the maximum luminescence and the area under the curve achieved between strains, the results resemble the ones obtained by immunoblot of Rps6, with the SA and WE strains having extreme phenotypes and the NA and WA strains having similar intermediate phenotypes (compare Figure 4C at time 30 min versus Figure 3B). Moreover, this novel methodology seems to recapitulate the EGOC-independent activation of TORC1 observed by immunoblot of Sch9, because even though the WE strain exhibited a great activation 5 minutes after the glutamine pulse, it lacked a re-activation at 30 minutes, while the SA strain showed the greater activation at this time (compare Figure 4B at time 30 min versus Figure 3B), consistent with the previously described activation of TORC1 independent of EGOC (Stracka et al., 2014).

In general, our method is based on a transcriptional reporter, making unfavourable a direct comparison with the results obtained by detection of a post-translational modification by immunoblot. Additionally, the temporary window used in the microculture experiment is greater than the used for immunoblotting experiments (4 hours vs 30 minutes, respectively). Therefore, our method has limitations, for example, it can take more time to overcome the background noise in this type of experiments than in a Western blot; in addition, cells take more time to transcribe and then translate the luciferase protein than to directly phosphorylate Rps6 or Sch9. Thus, this longer temporary window is also consistent with a possible evaluation of EGOC-independent activation of TORC1 by our method, because preferred nitrogen sources (like glutamine) are capable of a sustained TORC1 activation (Gonzalez & Hall, 2017; Stracka et al., 2014).

We corroborated that out method can detect the EGOC-independent activation of TORC1 by repeating the nitrogen upshift experiments using leucine, an amino acid that activates TORC1 only in a EGOC-dependent (Gtr1-dependent) manner and is unable of a sustained TORC1 activity (Stracka et al., 2014). The results showed that leucine is unable to increase the luminescence signal after a nitrogen upshift in the four strains evaluated, supporting the idea that our system only detect EGOC-independent activation of TORC1 by preferred nitrogen sources (Figure 5). We confirmed this result repeating the experiment in the SA and WE strains (the phenotypically more different strains) carrying *GTR1* deletion (*gtr1*Δ), confirming that this mutation has minimal effects on the TORC1 activation by a preferred nitrogen source such as glutamine (Figure 6A and C). Conversely, when we used leucine, a non-preferred nitrogen source, which activates TORC1 in an EGOC-dependent manner, we did not observe an increase in the reporter gene expression in the WT and *gtr1*Δ strains (Figure 6B and D). Altogether, these results are consistent with the idea that our system is capable to indirectly detect the EGOC-independent activation of TORC1, which occurs only by preferred nitrogen sources such as glutamine.

In general, the destabilized version of the firefly luciferase reporter gene has become an ideal tool to assess gene expression dynamics in living cells, allowing to measure the transcriptional activity of genes regulated by nutrient availability, osmotic stress and oxidative stress in yeasts (Dolz-Edo et al., 2013; Rienzo et al., 2015). In this sense, we used the luciferase reporter gene to record the transcriptional activity of *RPL26A* gene –which gene is known to be expressed upon TORC1 activation– using nitrogen upshift experiments, results that showed strong activation *RPL26A* only by a preferred nitrogen source (glutamine).

In conclusion, we report a new method based on growth under microculture conditions and using the luciferase reporter gene for indirect measuring of TORC1 EGOG-independent activity, being its results in partial agreement with those obtained through traditional

methodologies based on the relative estimation of the phosphorylation of Sch9 and Rps6 by immunoblot. The results obtained indicate that there are phenotypic differences in the kinetics of TORC1 activation by glutamine between distinct strains representative of *S. cerevisiae* clean lineages, with greater differences between Y12 (SA) and DBVPG6765 (WE) strains. This opens the possibility to use this new methodology to investigate the molecular basis of TORC1 activation by different nitrogen sources using high throughput approaches, like BSA, linkage analysis or comparative genomics, which requires phenotyping of numerous strains.

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Table 1. Yeast strains used in this work.

Name	Relevant genotype	Source/Reference
YPS128 a ('NA a')	*Mat a, ho::HygMX, ura3::KanMX*	(Cubillos et al., 2009)
Y12 a ('SA a')	*Mat a, ho::HygMX, ura3::KanMX*	(Cubillos et al., 2009)
DBVPG6044 a ('WA a')	*Mat a, ho::HygMX, ura3::KanMX*	(Cubillos et al., 2009)
DBVPG6765 a ('WE a')	*Mat a, ho::HygMX, ura3::KanMX*	(Cubillos et al., 2009)
YPS128 α ('NA α')	*Mat α, ho::NatMX, ura3::KanMX*	(Cubillos et al., 2009)
Y12 α ('SA α')	*Mat α, ho:: NatMX, ura3::KanMX*	(Cubillos et al., 2009)
DBVPG6044 α ('WA α')	*Mat α, ho:: NatMX, ura3::KanMX*	(Cubillos et al., 2009)
DBVPG6765 α ('WE α')	*Mat α, ho:: NatMX, ura3::KanMX*	(Cubillos et al., 2009)
YC022	YPS128 a pJU733	This work
YC023	Y12 a pJU733	This work
YC024	DBVPG6044 a pJU733	This work
YC025	DBVPG6765 a pJU733	This work
YC027	YPS128 a *rpl26a*::*Luc-URA3*	This work
YC028	Y12 a *rpl26a*::*Luc-URA3*	This work
YC029	DBVPG6044 a *rpl26a*::*Luc-URA3*	This work
YC030	DBVPG6765 a *rpl26a*::*Luc-URA3*	This work
YC031	YPS128 α *rpl26a*::*Luc-URA3*	This work
YC032	Y12 α *rpl26a*::*Luc-URA3*	This work
YC033	DBVPG6044 α *rpl26a*::*Luc-URA3*	This work
YC034	DBVPG6765 α *rpl26a*::*Luc-URA3*	This work
YC043	YC028xYC034	This work
YC231	YC032 g*tr1*::*HygMX*	This work
YC233	YC034 g*tr1*::*HygMX*	This work

Table 2. Growth parameters for WT strains and its version carrying *RPL26A* deletion.

Strain	Efficiency ± SD	Rate ± SD (h^{-1})	Lag ± SD (h)
NA (WT)	1.582 ± 0.039	0.081 ± 0.001	11.05 ± 0.39
NA (*rpl26a*Δ)	1.542 ± 0.016 ^{ns}	0.070 ± 0.005 *	12.48 ± 0.67 *
SA (WT)	1.607 ± 0.011	0.081 ± 0.004	13.18 ± 0.47
SA (*rpl26a*Δ)	1.626 ± 0.010 *	0.078 ± 0.004 ^{ns}	12.66 ± 0.29 ^{ns}
WA (WT)	1.518 ± 0.018	0.060 ± 0.006	19.95 ± 0.59
WA (*rpl26a*Δ)	1.531 ± 0.039 ^{ns}	0.053 ± 0.002 ^{ns}	18.11 ± 1,07 *
WE (WT)	1.440 ± 0.038	0.076 ± 0.004	12.61 ± 0.28
WE (*rpl26a*Δ)	1.425 ± 0.007 ^{ns}	0.087 ± 0.002 *	12.65 ± 0.42 ^{ns}

*: *rpl26a*Δ value significantly different form WT value (p-value < 0.05). ^{ns}: *rpl26a*Δ value not significantly different form WT value (p-value ≥ 0.05). SD: standard deviation.

Table 3. Parameters associated with luminescence curves.

Strain	$Max_{0\text{-}12}$ ± SD (a.u.)	$Time_{0\text{-}12}$ ± SD (h)	$AUC_{0\text{-}12}$ ± SD (a.u.)	$Max_{0\text{-}4}$ ± SD (a.u.)	$Time_{0\text{-}4}$ ± SD (h)	$AUC_{0\text{-}4}$ ± SD (a.u.)
NA	309 ± 50	5.2 ± 0.6	1230 ± 176	234 ± 44	2.4 ± 0.6	494 ± 40
SA	410 ± 7	1.7 ± 0.3	1324 ± 113	410 ± 7	1.7 ± 0.3	930 ± 66
WA	296 ± 43	3.5 ± 2.9	1408 ± 313	257 ± 13	2.3 ± 0.9	622 ± 44
WE	289 ± 14	8.4 ± 0.3	1213 ± 126	121 ± 8	2.3 ± 0.5	273 ± 36

Max: maximum luminescence. Time: maximum luminescence time. AUC: area under the curve of luminescence. $_{0\text{-}12}$: 0-12 hours' interval. $_{0\text{-}4}$: 0-4 hours' interval. SD: standard deviation.

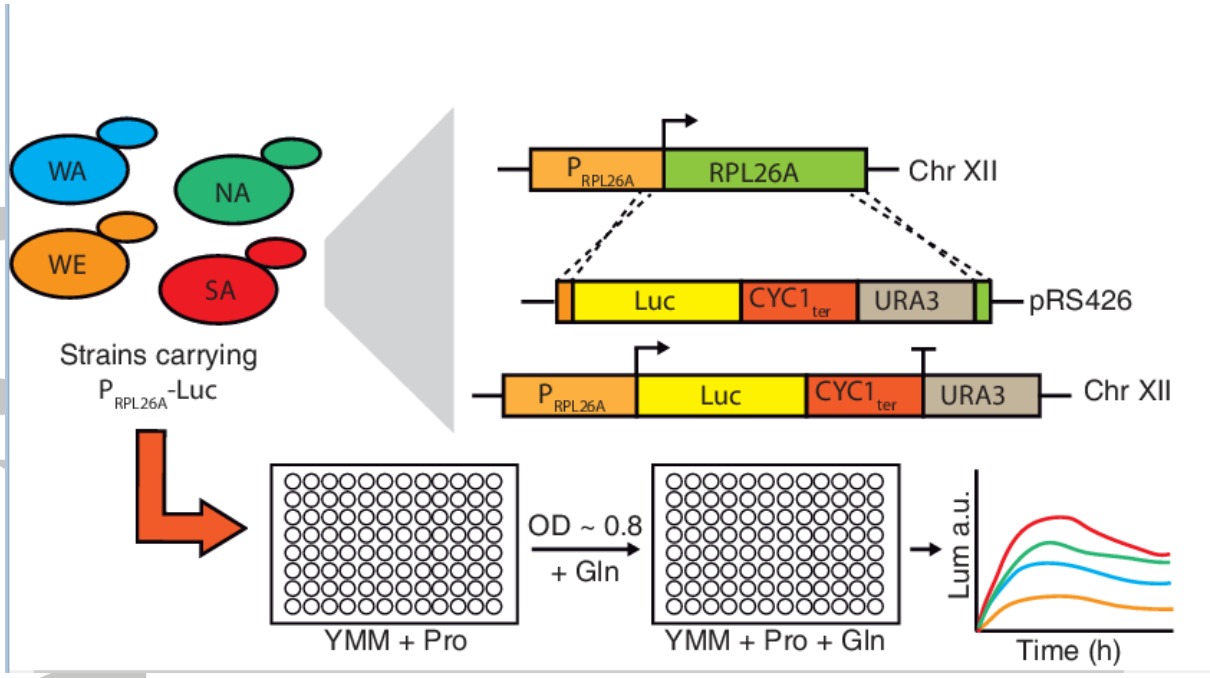

Figure 1. Method overview for indirect monitoring TORC1 activation in different yeast strains. The *RPL26A* ORF was replaced by the firefly luciferase, which is carrying the cytochrome C transcriptional terminator (*CYC1*$_{ter}$) and *URA3* as selectable marker. This strategy allows luciferase expression under the endogenous *RPL26A* promoter (*P*$_{RPL26A}$) control and without copy number bias. The four representative strains YPS128 (North American, 'NA'), Y12 (Sake, 'SA'), DBVPG6044 (West African, 'WA') and DBVPG6765 (Wine/European, 'WE') were evaluated in a nitrogen upshift experiment, growing them in a 96-well plate with YMM plus proline (YMM+Pro) medium an adding glutamine (Gln) as nitrogen source when the strains reached OD_{600} ~0.8. The luminescence (Lum) of the cells was recorded after the nitrogen pulse every 5 minutes.

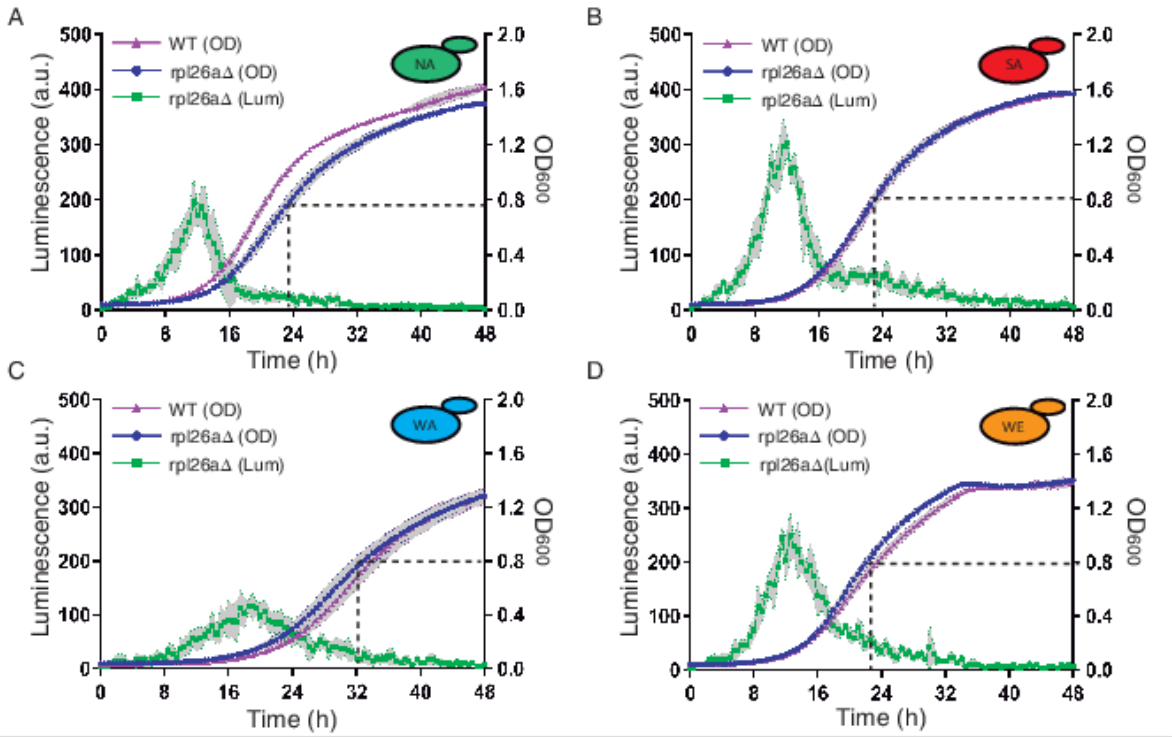

Figure 2. Growth and luciferase expression for strains carrying the reporter construct. The strains **(A)** NA, **(B)** SA, **(C)** WA and **(D)** WE were transformed with the *Luc-URA3* construct replacing the *RPL26A* ORF. These strains (*rpl26a*Δ) and their wild type (WT) versions were grown in YMM+Pro medium plus luciferin in microculture conditions, monitoring the OD_{600} and the luminescence (Lum) of the yeast cells over time. Black dashed lines show the time at which an OD_{600} ~0.8 was reached by strains carrying the luciferase. Plotted values correspond to the average of three biological replicates, with their standard error represented by shadow regions (mean ± SEM).

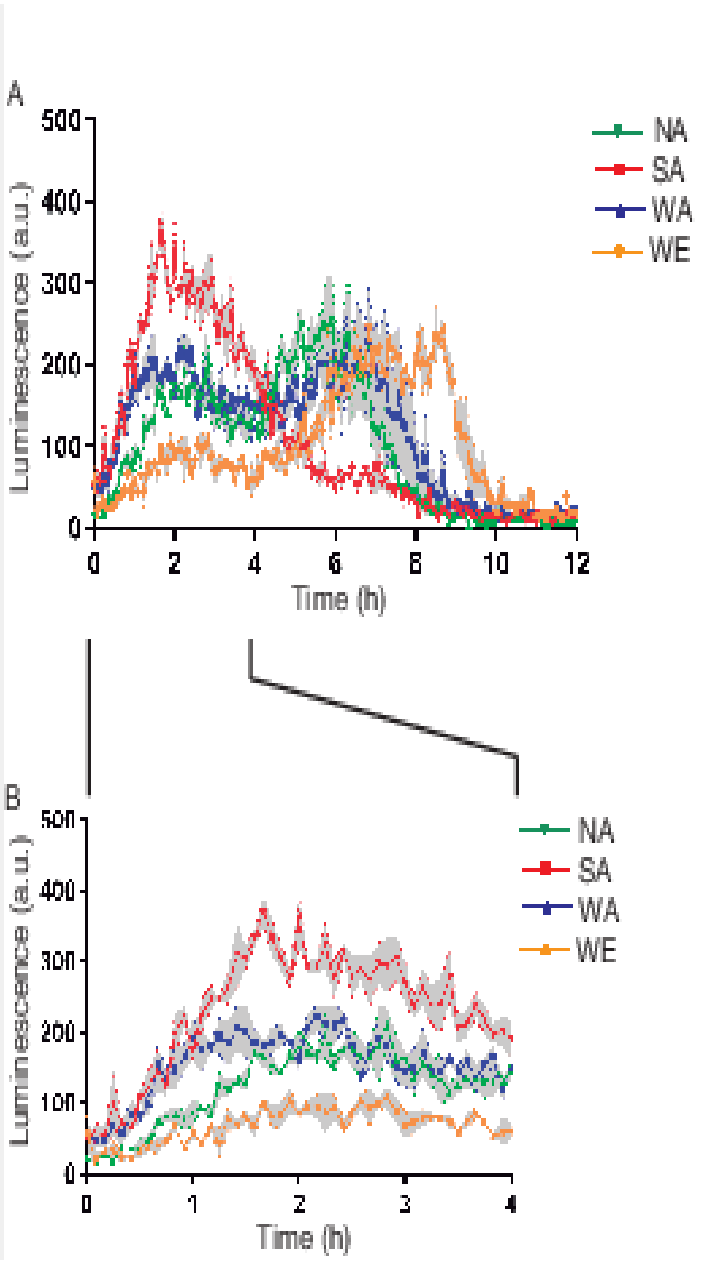

Figure 3. Nitrogen upshift experiment using the new method developed. Luminescence (Lum) differences between strains were evaluated in microculture conditions after a pulse of glutamine. The luminescence was recorded until 12 hours after nitrogen pulse **(A)**. A zoom in of the first four hours after the nitrogen (proline-to-glutamine) upshift experiment (**B**). In both panels (A and B), the time 0 h corresponds to the addition of glutamine. Plotted values correspond to the average of three biological replicates, with their standard error represented by shadow regions (mean ± SEM).

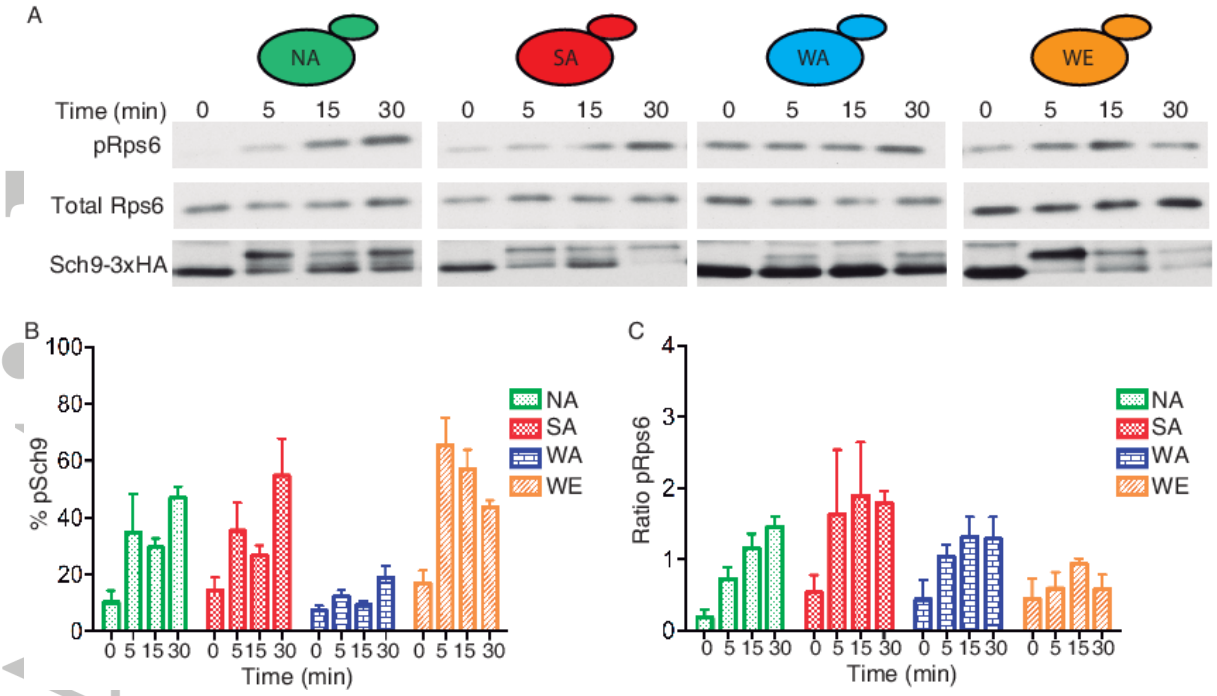

Figure 4. Measuring TORC1 activity by immunoblot. (A) Western blot for the strains NA, SA, WA and WE. In the case of Rps6, phosphorylated (pRps6) and total Rps6 protein levels were evaluated using specific antibodies. In the Sch9 phosphorylation, an antibody that recognizes the 3xHA epitope was used, which gives an upper and a lower band that are representative of phosphorylated (pSch9) and non-phosphorylated (non-pSch9) isoforms, respectively. Time 0 min corresponds to the addition of glutamine. **(B)** Quantification of Sch9 phosphorylation. The "% pSch9" is the ratio between the pSch9 band intensity and the sum of the pSch9 and non-pSch9 band intensities, multiplied by 100. Plotted values correspond to the average of three biological replicates, with their standard error represented by bars (mean ± SEM). **(C)** Quantification of Rps6 phosphorylation. The "Ratio pRps6" is the ratio between pRps6 band intensity and the total Rps6 band intensity, normalized by the mean value of all the ratios obtained. Plotted values correspond to the average of three biological replicates, with their standard error represented by bars (mean ± SEM).

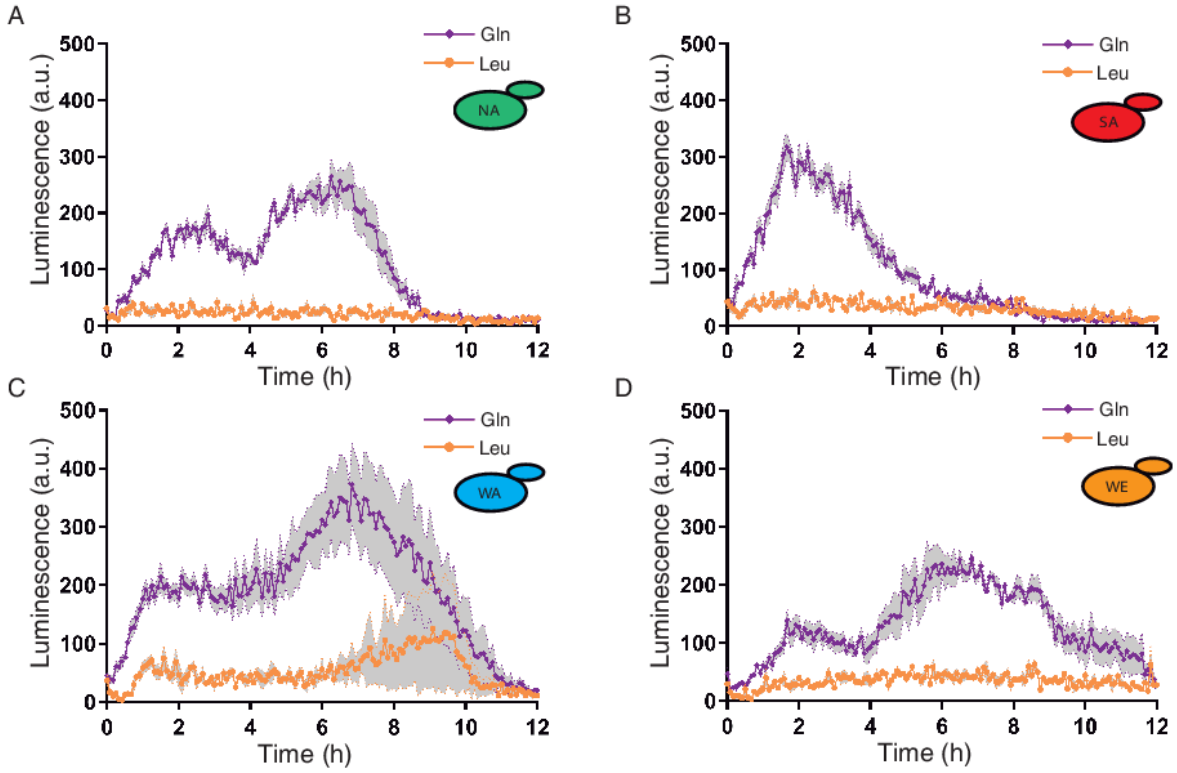

Figure 5. Nitrogen upshift experiment using glutamine and leucine. Luminescence (Lum) differences between strains **(A)** NA, **(B)** SA, **(C)** WA and **(D)** WE were evaluated in microculture conditions after a pulse of glutamine (Gln) and a pulse of leucine (Leu). The luminescence was recorded until 12 hours after nitrogen pulse. Time 0 h corresponds to the addition of glutamine or leucine. Plotted values correspond to the average of three biological replicates, with their standard error represented by shadow regions (mean ± SEM).

Figure 6. Nitrogen upshift experiment using *gtr1*Δ strains. Luminescence (Lum) differences between strains SA **(A and B)** and WE **(C and D)** were evaluated in microculture conditions after a pulse of glutamine **(A and C)** or leucine **(B and D)**. The luminescence was recorded until 12 hours after nitrogen pulse. Time 0 h corresponds to the addition of glutamine or leucine. Plotted values correspond to the average of three biological replicates, with their standard error represented by shadow regions (mean ± SEM).