Pas1, a G1 cyclin, regulates amino acid uptake and rescues a delay in G1 arrest in Tsc1 and Tsc2 mutants in Schizosaccharomyces pombe

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Tuberous sclerosis complex is a tumor suppressor syndrome caused by mutations in either the TSC1 or the TSC2 gene. Previous studies have shown that deletion of the TSC1 or TSC2 ortholog in Schizosaccharomyces pombe results in an amino acid uptake defect, with conditional lethality. We identified a G1 cyclin, pas1+, as a high-copy suppressor of this defect in Δtsc1. Disruption of pas1+ causes defects in arginine and leucine uptake that are remarkably similar to Δtsc1 and Δtsc2, whereas Δpas1Δtsc1 and Δpas1Δtsc2 double mutants have more severe amino acid uptake defects. In a second screen, we identified a novel G63D/S165N mutant of the small GTPase Rhb1, the target of the Tsc1/Tsc2 protein complex. The Rhb1 mutant suppresses amino acid uptake in Δtsc1 yeast, but not in Δpas1 yeast. Hence, Pas1 does not regulate amino acid uptake through Rhb1. To determine whether Pas1 links nutrient availability to cell cycle progression downstream of the Tsc1/Tsc2 complex, we examined the kinetics of G1 arrest in single and double mutant strains. After nitrogen starvation, Δtsc1 and Δtsc2 yeast had a delay in G1 arrest compared with wild-type, which was rescued by deletion of pas1+. In summary, we identified the G1 cyclin, Pas1, as a novel regulator of amino acid uptake. Our data support a model in which Pas1 inhibits G1 arrest downstream of Tsc1 and Tsc2, linking nutrient uptake and cell cycle progression in yeast.

INTRODUCTION

Tuberous sclerosis complex (TSC) is a tumor suppressor gene disorder characterized by benign tumors (hamartomas), lymphangiomyomatosis and severe neurological problems including seizures, mental retardation and autism. TSC is caused by mutations in either TSC1 (1) or TSC2 (2). The protein products of TSC1 and TSC2 interact (3,4) and function as a complex. Studies in Drosophila and mammalian systems have shown that the small GTPase Rheb (Ras homolog enriched in brain) is a direct downstream target of the TSC1–TSC2 complex (5–11). Rheb binds and activates target of rapamycin (TOR) (12–14), an evolutionarily conserved kinase that integrates signals from nutrients (amino acids and energy) and growth factors (insulin) to regulate cell growth, cell cycle progression and nutrient uptake (15–17). Although many studies have focused on the downstream effectors of TSC1/TSC2 and TOR, little is known about the role of the TSC1/TSC2 complex in amino acid uptake and cell cycle regulation.

Schizosaccharomyces pombe contains genes with significant homology to mammalian TSC1, TSC2, RHEB and TOR, which are referred to as tsc1+, tsc2+, rhb1+, tor1+ and tor2+, respectively. We and others have previously shown that strains lacking tsc1+ and tsc2+ (Δtsc1 and Δtsc2) have defects in the uptake of arginine and leucine. This amino acid uptake defect results in a conditional lethal phenotype where growth of Δtsc1 and Δtsc2 is dependent on the concentrations of amino acids in the yeast growth medium (18,19). The defect in arginine uptake in cells lacking tsc2+ is rescued by expression of a dominant-negative form of rhb1+ (19) suggesting that Rhb1 is downstream of Tsc2 in S. pombe, as has been shown in Drosophila and mammals. Interestingly, loss of rhb1+ in S. pombe results in increased sensitivity to a toxic analog of arginine (20) and to a G1 growth arrest, similar to that caused by nitrogen starvation (21,22), suggesting a link between amino acid uptake and the cell cycle.

To identify downstream targets of the Tsc1 and Tsc2 proteins in S. pombe that mediate amino acid uptake, we performed a screen for genes whose overexpression rescues the
lethal growth phenotype in Δtsc1. This screen identified a G1 cyclin, pas1Δ, leading us to hypothesize that pas1Δ signals downstream of Tsc1 and Tsc2 in S. pombe to link nutrient availability to cell cycle progression. We found that Δpas1 yeast have defects in amino acid uptake similar to Δtsc1 and Δtsc2. However, our data suggest that the primary role of Pas1 is to inhibit G1 arrest downstream of the Tsc1/Tsc2 complex in S. pombe. Our study therefore provides new insight into the molecular mechanisms linking amino acid uptake and cell cycle progression.

**RESULTS**

**Pas1 rescues the conditional lethal growth phenotype of Δtsc1**

We and others previously found that deletion of tsc1Δ in the CHP429 (h+, leu1-32, ura4-D18, ade6-216, his7-366) strain (referred to as F15Δtsc1) results in a conditionally lethal phenotype on essential minimal medium (EMM) supplemented with 50 mg/l of uracil, adenine, leucine and histidine, due to defects in amino acid uptake (18,19). Growth can be partially restored when the amounts of supplements are increased. To identify downstream targets of the Tsc1/Tsc2 complex that mediate this phenotype, we screened for high-copy suppressors that restore growth on 50 mg/l of uracil, adenine and histidine. Sequence analysis indicated that tsc1Δ was among the rescuers, serving as a positive control. One of the other rescuers contained a complete open-reading frame of SPAC19E9.03, also referred to as pas1Δ. The pas1Δ gene was of particular interest because it encodes a putative G1 cyclin in S. pombe (23).

We first confirmed the rescue in F15Δtsc1 by transformation of the original genomic fragment from the library containing the full-length pas1Δ gene. Although pas1Δ was identified as a rescuer of growth for F15Δtsc1, it also restored growth to a similar degree in F15Δtsc2 (Fig. 1A).

We had previously observed in a microarray experiment that pas1Δ mRNA was upregulated 1.8-fold in the Δtsc1 strain compared with wild-type yeast (unpublished data). This led us to ask whether pas1Δ mRNA expression is also upregulated in Δtsc2. We found that the mRNA expression of pas1Δ was increased in Δtsc1 and Δtsc2 at least 2-fold. In contrast, tsc1Δ and tsc2Δ expressions were not significantly changed in the Δpas1 strain (Fig. 1B).

**Δpas1 has amino acid uptake defects similar to Δtsc1 and Δtsc2**

Tsc1 and Tsc2 are known to regulate arginine and leucine uptake in S. pombe (18,19). The arginine uptake defect results in resistance to 60 mg/l canavanine, a toxic analog of arginine. We found that the Δpas1 strain was also resistant to 60 mg/l canavanine, similar to Δtsc1 and Δtsc2 (Fig. 2A). To determine whether Pas1 regulates amino acid uptake downstream of Tsc1/Tsc2, we crossed Δpas1 with Δtsc2 and with Δtsc1 to generate Δpas1Δtsc1 and Δpas1Δtsc2 double mutant strains. We predicted that if Pas1 and Tsc1/Tsc2 are in the same pathway, the Δpas1Δtsc1 and Δpas1Δtsc2 double mutant strains would have equally impaired amino acid uptake as the single mutants. We found that the Δpas1Δtsc1 and Δpas1Δtsc2 double mutants were more resistant to canavanine than the single mutants, as reflected by enhanced growth on plates containing 60 mg/l canavanine (Fig. 2A). These results suggest that Pas1 and the Tsc1/Tsc2 complex regulate arginine uptake through parallel pathways. To determine whether the canavanine resistance in the Δpas1 strain was due to decreased arginine uptake by the mutant strains, uptake of 3H-arginine was measured. Arginine uptake was significantly reduced in the Δpas1 strain (2.5-fold, P < 0.05) compared with wild-type (Fig. 2B), which is equivalent to Δtsc1 and Δtsc2. The Δpas1Δtsc1 and Δpas1Δtsc2 double mutants were more severely affected (10-fold reduction of arginine uptake) than the Δpas1, Δtsc1 or Δtsc2 single mutants (P < 0.05) (Fig. 2B). These results show that canavanine resistance in the Δpas1, Δtsc1 and Δtsc2 strains is correlated with arginine uptake defects.

Next, we tested whether Δtsc1, Δtsc2 and Δpas1 yeast are resistant to DL-ethionine, a toxic analog of methionine in S. pombe. We found that the Δtsc1, Δtsc2 and Δpas1 strains are resistant to 30 mg/l DL-ethionine. The Δpas1Δtsc1 and Δpas1Δtsc2 double mutant strains are more resistant to DL-ethionine than the single mutants (Fig. 2A), similar to our findings with canavanine. These data suggest that the uptake of multiple amino acids is affected by inactivation of Pas1 or the Tsc1/Tsc2 complex.

Because it was shown before that Tsc1 and Tsc2 regulate leucine uptake in S. pombe (18), we compared 3H-leucine uptake in the Δpas1, Δtsc1 and Δtsc2 single mutants and the Δpas1Δtsc1 and Δpas1Δtsc2 double mutants. We found that leucine uptake was reduced ~2.5-fold (P < 0.05) in Δpas1 relative to wild-type, which is comparable to Δtsc1 and Δtsc2 (Fig. 2C). The Δpas1Δtsc2 double mutants were again more severely affected than Δpas1 (P < 0.05), Δtsc1 (P < 0.05) or Δtsc2 (P < 0.05) single mutants (Fig. 2C). Taken together, these data demonstrate...
that Pas1 and the Tsc1/Tsc2 complex regulate amino acid uptake. The additive effect for each phenotype in the double mutants suggests that Pas1 and the Tsc1/Tsc2 complex have independent and synergistic effects on amino acid uptake.

**Δpas1** has decreased expression of amino acid permeases

We previously found that the mRNA expression of three amino acid permeases (SPAC869.10\(^+\), SPAP7G5.06\(^+\) and isp5\(^+\)) is reduced in the S. pombe Δtsc1 and Δtsc2 strains...
non-conserved residue within a conserved region. Human and S. pombe sequences, whereas the S165 N substitution is at a residue that is identical in the S. pombe and humans, consistent with our previous study, and also by deletion of pas1 +, consistent with our previous finding that Rhb1 is the target gene in all three clones. We identified three clones (A2, A3 and A4) cells were spotted on EMM plates (left panel) and on EMM plates supplemented with 50 mg/l of adenine, leucine, histidine and uracil (left panel) and with canavanine (right panel). Plates were incubated at 30°C for 3 days and then photographed.

Pas1 overexpression partially restores the arginine uptake defect in Δtsc1 and Δtsc2

We next investigated whether overexpression of pas1 + could rescue the canavanine resistance of Δtsc1, Δtsc2 and Δpas1 mutants. As expected, the canavanine sensitivity in the Δpas1 strain was restored by expression of pas1 +. Furthermore, expression of pas1 + restored canavanine sensitivity in the Δtsc1 and Δtsc2 strains almost to the level detected with tsc1 + or tsc2 + overexpression (Fig. 2E). We next measured radiolabeled arginine uptake when pas1 + was overexpressed in the Δtsc1 or Δtsc2 strain. Using this assay, we found that arginine uptake in Δtsc1 or Δtsc2 with overexpression of pas1 + was ~50% restored (Fig. 2F). Taken together, these results suggest that arginine uptake in S. pombe is regulated through Tsc1/Tsc2 and Pas1 via independent pathways.

RhbG63DS165N mutant can restore canavanine sensitivity in Δtsc1

To complement our overexpression screen, we performed a second screen for mutations in endogenous genes that rescue the growth defect in F15Δtsc1 on 50 mg/l of supplements. We identified three clones (F15Δtsc1A2, F15Δtsc1A3 and F15Δtsc1A4) that could grow on minimal medium with 50 mg/l supplements. The F15Δtsc1A2 strain was sensitive to canavanine, whereas F15Δtsc1A3 and F15Δtsc1A4 were resistant to canavanine (Fig. 3A), suggesting that the mutation in the F15Δtsc1A2 strain rescued arginine uptake. Because we expected that dominant-negative or partial loss-of-function mutations in Rhb1 would rescue defects in Δtsc1, we sequenced the rhb1 + gene in all three clones. We identified two mutations in the rhb1 + gene in F15Δtsc1A2, G63D and S165 N. No rhb1 + mutations were identified in F15Δtsc1A3 or F15Δtsc1A4. Human and S. pombe Rheb are 52% identical at the amino acid level and an additional 35% of the residues are conserved changes (Fig. 3B). The G63D substitution occurs at a residue that is identical in the S. pombe and human sequences, whereas the S165 N substitution is at a non-conserved residue within a conserved region.

To study the effect of the Rhb1G63DS165N mutant on arginine uptake in Δpas1 and Δtsc2, we crossed F15Δtsc1A2 with the wild-type, Δtsc2 and Δpas1 strains to generate Rhb1G63DS165N Δtsc1/Δtsc2 Rhb1G63DS165N Δtsc1 Δpas1Δtsc2 Δtsc1 Δtsc2 Rhb1G63DS165N and Δpas1 Rhb1G63DS165N strains. After crossing, the presence of the Rhb1G63DS165N mutations in all clones was verified by sequencing. We were not able to generate Rhb1G63DS165N in combination with Δtsc2, suggesting that this genotype is lethal. We tested the effect of the Rhb1G63DS165N mutant on canavanine sensitivity in yeast lacking either Tsc1 or Pas1. We found that Rhb1G63DS165N restored canavanine sensitivity in the Δtsc1 strain, suggesting that Rhb1G63DS165N is a novel partial loss-of-function mutant, and confirmed our previous finding that Rhb1 is the target of the Tsc1/Tsc2 complex in S. pombe. However, Rhb1G63DS165N did not restore canavanine sensitivity in the Δpas1 strain (Fig. 3C), suggesting that Pas1 does not signal through Rhb1 to regulate amino acid uptake in S. pombe.
**Δtsc1** and **Δtsc2** have a delay in G1 arrest, which is rescued by deletion of **pas1**

Because Pas1 was originally identified in a search for new factors controlling the cell cycle start in *S. pombe* (23), we asked whether **Δtsc1** and **Δtsc2** yeast have a defect in G1 arrest in *S. pombe*. G1 arrest in *S. pombe* is induced by nitrogen starvation and results in one-copy peak in the cell cycle profile. When compared with wild-type yeast, the **Δtsc1** and **Δtsc2** yeast had a delay in G1 arrest after 4 and 8 h of nitrogen starvation (Fig. 4A). We next tested whether nitrogen-induced G1 arrest was also affected in the **Δpas1Δtsc1** and **Δpas1Δtsc2** double mutants. Deletion of **pas1** in **Δtsc1** or **Δtsc2** restored the delay in nitrogen-induced G1 arrest observed in the **Δtsc1** or **Δtsc2** single mutants (Fig. 4A). This was unexpected, because **pas1** was identified in a screen for genes that by overexpression could rescue growth in F15**Δtsc1** yeast.

Next, we tested the effect of overexpression of **pas1**, **tsc1** or **tsc2** on the delay in nitrogen-induced G1 arrest in **Δtsc1** or **Δtsc2**. Exogenous expression of **tsc1** or **tsc2** rescued the delay in G1 arrest in **Δtsc1** or **Δtsc2**, respectively, but exogenous expression of **pas1** did not (Fig. 4B). Furthermore, exogenous expression of **tsc1**, **tsc2** or **pas1** induced a small G1 one-copy peak without nitrogen starvation, suggesting that Pas1, Tsc1 and Tsc2 regulate G1 arrest in *S. pombe* (Fig. 4B). The rescue of **Δtsc1** and **Δtsc2** by deletion of Pas1 strongly suggests that Pas1 functions downstream of Tsc1/Tsc2 to inhibit G1 arrest (Fig. 4C).

**DISCUSSION**

We previously found that Tsc1 and Tsc2 mutants in *S. pombe* have amino acid uptake defects, resulting in a conditional lethal phenotype where growth of **Δtsc1** and **Δtsc2** is dependent on the concentrations of amino acids in the yeast growth medium (19). To identify downstream effectors that might mediate this phenotype, we used a genetic screen approach.

We report here the identification of a G1 cyclin, **pas1**, as a suppressor of the growth defect in **Δtsc1** on low supplements. Disruption of **pas1** results in leucine and arginine uptake...
defects that are similar to the defects in \( \Delta tsc1 \) and \( \Delta tsc2 \). Importantly, \( \Delta tsc1 \Delta pas1 \) and \( \Delta tsc2 \Delta pas1 \) double mutants are more severely affected than \( \Delta tsc1 \), \( \Delta tsc2 \) or \( \Delta pas1 \) single mutants, suggesting that \( \Delta pas1 \) regulates amino acid uptake in a pathway that is parallel to Tsc1 and Tsc2. Consistent with these results, we found that overexpression of \( pas1^+ \) in \( \Delta tsc1 \) or overexpression of \( pas1^+ \) in \( \Delta tsc1 \) only partially rescued the resistance of \( \Delta tsc1 \) and \( \Delta tsc2 \) to canavanine, a toxic analog of arginine. The finding that \( pas1^+ \) mRNA was upregulated in \( \Delta tsc1 \) and \( \Delta tsc2 \) yeast suggests that all three proteins might be linked through a common downstream amino acid sensing mechanism.

Pas1 was originally identified as a multicopy suppressor of the \( res1 \) null mutant (23). \( res1 \) is part of the \( Res1–Cdc10 \) transcriptional regulator complex that together with the redundant \( Res2–Cdc10 \) complex activate genes that are essential for the \( G_1–S \) transition in mammalian cells. The \( Pas1 \) protein in \( S. pombe \) has also been linked to the \( G_1–S \) transition, with predicted orthologs in mammals, insects, worms and yeast, but nothing is known about its function in mammalian cells. The \( Pas1 \) protein in \( S. pombe \) and \( S. cerevisiae \) regulates amino acid uptake and cell cycle progression in response to nitrogen starvation, with evidence for a possible link between amino acid uptake and cell cycle regulation in \( S. pombe \) (24–26). We found that \( \Delta pas1 \) in \( S. pombe \) arrests the growth of the \( \Delta tsc1/\Delta tsc2 \) double mutant, suggesting a possible link between amino acid uptake and cell cycle progression in \( S. pombe \).

Overexpression of either \( TSC1 \) or \( TSC2 \) in mammalian or insect cells has also been linked to the \( G_1–S \) transition, either by lengthening \( G_1 \) or by inhibiting cell proliferation (24–26). We found that \( \Delta tsc1 \) and \( \Delta tsc2 \) cells have a delay in \( G_1 \) arrest in \( S. pombe \), which is not rescued by \( pas1^+ \) overexpression. However, deletion of \( pas1^+ \) in \( \Delta tsc1 \) or \( \Delta tsc2 \) yeast restored the \( G_1 \) arrest. This result was unexpected, because \( Pas1 \) was identified in an overexpression screen, but overexpression of either \( TSC1 \) or \( TSC2 \) in mammalian or insect cells has also been linked to the \( G_1–S \) transition.

Importantly, \( \Delta tsc1 \) or \( \Delta tsc2 \) are more severely affected than the \( \Delta res1 \) single mutants, suggesting that \( \Delta res1 \) regulates amino acid uptake in a pathway that is parallel to Tsc1 and Tsc2. Consistent with these results, we found that overexpression of \( res1^+ \) in \( \Delta tsc1 \) or overexpression of \( res1^+ \) in \( \Delta tsc1 \) only partially rescued the resistance of \( \Delta tsc1 \) and \( \Delta tsc2 \) to canavanine, a toxic analog of arginine. The finding that \( res1^+ \) mRNA was upregulated in \( \Delta tsc1 \) and \( \Delta tsc2 \) yeast suggests that all three proteins might be linked through a common downstream amino acid sensing mechanism.

In summary, our data demonstrate that a \( G_1 \) cyclin, \( Pas1 \), regulates arginine and leucine uptake in \( S. pombe \). The similarities between the \( \Delta pas1 \) and \( \Delta tsc1 \) and \( \Delta tsc2 \) strains suggest a possible link between amino acid uptake and cell cycle regulation in \( S. pombe \). In addition, our finding that \( \Delta tsc1 \) can be rescued by the novel \( Rhb1^{G63D} \) mutant supports previous findings that \( Tsc1/\Delta tsc2 \) are upstream of \( Rhb1 \) in \( S. pombe \). Finally, we found for the first time that \( \Delta tsc1 \) and \( \Delta tsc2 \) yeast have a delay in nitrogen starvation-induced \( G_1 \) arrest, which is rescued by deletion of \( pas1^+ \), indicating that \( Pas1 \) inhibits \( G_1 \) arrest downstream of the \( Tsc1/\Delta tsc2 \) complex.

## MATERIALS AND METHODS

### Yeast strains, media and growth conditions

The yeast strains used in this study are listed in Table 1. The \( \Delta res1 \) mutant strain, K193-A1, was a gift from Koichi Tanaka (University of Tokyo). Wild-type strain 972 (28) was a gift from Norbert Käuf er (TU Braunschweig). Yeast transformations were performed with the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, USA). After 5 days of growth, plasmids were

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### High-copy suppressor screen and plasmid isolation

The pAL18 ssp41 Cs2-21 yeast genomic library, with insert size averaging \( \sim 2.3 \) kb, was a gift from Norbert Käuf er (TU Braunschweig). Yeast transformations were performed with the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, USA). After 5 days of growth, plasmids were
isolated from colonies that had formed on EMM supplemented with 50 mg/l of uracil, adenine and histidine. pAL18 plasmids containing full-length \(tsc1^+\), \(tsc2^+\) and \(pas1^+\) were used in all transformation studies.

Construction of \(\Delta tsc1\Delta pas1\) and \(\Delta tsc2\Delta pas1\) double mutant strains

\(Tsc1^+\) and \(tsc2^+\) deficient strains MVS1, MVS2, MVS3, MVS4, MVS5 (F15\(tsc1\)) and MVS6 (F15\(tsc2\)) were constructed as described before (19). MVS3 and MVS4 were mated on malt extract medium with K193-A1 to generate MVS1P (referred to as \(\Delta tsc1\Delta pas1\)) and MVS2P (referred to as \(\Delta tsc2\Delta pas1\)) double mutant strains. After 2 days of mating, ascis with spores were treated with snail enzyme for 6 h and subjected to random spore analysis on plates with G418 to select for \(\Delta tsc1\), followed by EMM with no uracil to select for \(\Delta pas1\). The double mutants were verified by PCR and loss of expression was determined by northern blot analysis.

Northern blot analysis

RNA was isolated by phenol extraction and 10 \(\mu\)g of total RNA was run on a 1% formaldehyde gel at 100 V for 2 h and transferred to nylon membrane overnight in 20\(\times\) SSC. Probes for \(pas1^+\), \(tsc2^+\), e689.10, 7G5.06, isp5 and gpd3 were PCR amplified from cDNA, cleaned over 0.8% agarose gel and labeled with [\(\alpha-32P\)]dCTP (Perkin Elmer, Wellesley, MA, USA) using standard methods. Hybridizations were performed in rapid hybridization buffer (Amersham Biosciences, Piscataway, NJ, USA).

Amino acid analog sensitivity

Cells were grown overnight to midlog phase (OD 595\,\text{nm} = 0.4–0.6), and OD 595 was adjusted to 0.4 (10 000 cells/\(\mu\)l). About 4\(\mu\)l of 10\(\times\), 100\(\times\) and 1000\(\times\) dilution was spotted onto EMM as a growth control, or EMM containing canavanine (60 \(\mu\)g/ml) or DL-ethionine (30 \(\mu\)g/ml) (Sigma, St Louis, MO, USA) and incubated for 3 days at 30\(^\circ\)C.

Radiolabeled amino acid uptake assays

Arginine and leucine uptake assays were performed in triplicate as described by Urano et al. (29), with minor modifications. Cells were grown in EMM minimal medium with no supplements to midlog phase. One microcurie of L-\(^3\)H-labeled amino acid (40–70 Ci/mmole) (Perkin Elmer) and 100 \(\mu\)M of non-radioactive amino acid (Sigma) were added to 25 000 cells in 600 \(\mu\)l of EMM. Aliquots of 200 \(\mu\)l were removed at 0 and 10 min, injected into 5 ml of deionized water and immediately subjected to vacuum manifold filtration. Cells were collected on Whatman glass microfiber filters, washed twice and dried. \(^{3}\)H-arginine and \(^{3}\)H-leucine were measured by scintillation counting.

Flow cytometry

Cells were grown for 16 h in EMM until midlog phase. Cells were washed twice with \(H_2O\) and resuspended in EMM without nitrogen (Qbiogene). Cells were then grown for 4 h at 25\(^\circ\)C to induce \(G_1\) arrest. Cells were collected and fixed with 70% ice-cold ethanol. For FACS analysis, cells were resuspended in 50 mm sodium citrate, washed in the same buffer and treated with RNase for 16 h. Before analysis, the yeast cells were briefly sonicated and stained with propidium iodide at a final concentration of 16 \(\mu\)g/ml. Thirty thousand cells were collected with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed with WINMDI 2.8 software (www.facs.scripps.edu).

Mutagenesis screen

Cells were grown till midlog phase, washed in TM buffer (50 mm Tris-maleate, pH 6.0) and resuspended in TM at 3.5 \(\times\) 10\(^8\) cells/ml. About 350 \(\mu\)l of cell suspension was mixed with 150 \(\mu\)l of nitrosoguanidine NG (1 mg/ml in TM) and left at 30\(^\circ\)C for 8 min to induce 30–50% cell survival (determined by kill curve). Fifty microliters of mutagenized cells were washed three times with 1 ml TM and plated onto EMM supplemented with 50 mg/l of uracil, leucine, adenine and histidine.

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Conflict of Interest statement. None declared.

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