Fission yeast Ryh1 GTPase activates TOR Complex 2 in response to glucose

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The Target Of Rapamycin (TOR) is an evolutionarily conserved protein kinase that forms 2 distinct protein complexes referred to as TOR Complex 1 (TORC1) and 2 (TORC2). Recent extensive studies have demonstrated that TORC1 is under the control of the small GTPases Rheb and Rag that funnel multiple input signals including those derived from nutritional sources; however, information is scarce as to the regulation of TORC2. A previous study using the model system provided by the fission yeast Schizosaccharomyces pombe identified Ryh1, a Rab-family GTPase, as an activator of TORC2. Here, we show that the nucleotide-binding state of Ryh1 is regulated in response to glucose, mediating this major nutrient signal to TORC2. In glucose-rich growth media, the GTP-bound form of Ryh1 induces TORC2-dependent phosphorylation of Gad8, a downstream target of TORC2 in fission yeast. Upon glucose deprivation, Ryh1 becomes inactive, which turns off the TORC2-Gad8 pathway. During glucose starvation, however, Gad8 phosphorylation by TORC2 gradually recovers independently of Ryh1, implying an additional TORC2 activator that is regulated negatively by glucose. The paired positive and negative regulatory mechanisms may allow fine-tuning of the TORC2-Gad8 pathway, which is essential for growth under glucose-limited environment.

Introduction

The Target of Rapamycin (TOR) is an evolutionarily conserved serine/threonine kinase that forms 2 distinct protein complexes referred to as TOR Complex 1 (TORC1) and 2 (TORC2).1-5 While TORC1 and TORC2 share the catalytic subunit TOR kinase, their specific regulatory subunits confer discrete physiological functions to the 2 complexes. Thus, TORC1 and TORC2 control distinct sets of downstream targets under different regulations.6-13

To elucidate the function and regulation of each TOR complex, extensive studies have been conducted in a variety of model organisms. Among those is the fission yeast Schizosaccharomyces pombe, a genetically amenable eukaryote with nonessential TORC2, which eases mutational analysis of this particular TOR complex.14-21 Fission yeast TORC2 is composed of the catalytic subunit Tor1 kinase and the regulatory subunits named Ste20, Sin1, Wat1 and Bit61,17,19,22 The key downstream target of TORC2 in fission yeast is the AGC-family kinase Gad8, as evidenced by the observation that the gad8 deletion mutant phenocopies the TORC2-defective mutants.16,19 Like Akt activation by mammalian TORC2, Gad8 is phosphorylated within its hydrophobic motif by TORC2, resulting in increased Gad8 kinase activity essential for its cellular functions.16,19 On the other hand, a major upstream regulator of fission yeast TORC2 has been found as the Rab small GTPase Ryh1.21 The GTP-bound, active form of Ryh1 physically interacts with TORC2 and promotes TORC2-dependent phosphorylation/activation of the Gad8 kinase.

In diverse eukaryotic species, TORC1 controls cellular anabolic and catabolic processes, such as translation and autophagy, in response to nutrients and other extracellular stimuli.6,23-31 Fission yeast TORC1 is activated in the presence of nitrogen and carbon sources, and phosphorylates the major S6 kinase Psk1.22,32-34 In this organism, TORC2 is also implicated in cellular nutritional responses, including the G1 arrest upon nitrogen starvation and the mitotic control in response to glucose.16,19,35 Little is known, however, about the physiological stimuli that regulate fission yeast TORC2, whereas insulin and growth factors activate mammalian TORC2 (mTORC2) in a PI3K-dependent manner.7,8,36-38

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In this report, we show that TORC2 in fission yeast is responsive to glucose and that the functional TORC2-Gad8 pathway is essential for cell proliferation in glucose-limited environment. Our mutational analysis indicated that the glucose regulation of fission yeast TORC2 is not dependent on the cAMP pathway, a signaling mechanism known to be responsive to extracellular glucose. Importantly, glucose-replete conditions increase the active, GTP-bound form of Ryh1 GTPase, which induces TORC2-dependent phosphorylation of Gad8. Glucose starvation swiftly inactivates Ryh1 and suppresses the TORC2-Gad8 pathway, which is, however, gradually reactivated in a manner independent of Ryh1. Thus, the fission yeast TORC2-Gad8 pathway appears to be controlled by Ryh1-dependent and -independent mechanisms that monitor the availability of glucose, a major carbon/energy source for fission yeast.

**Results**

**TORC2 remains active during nitrogen starvation**

When starved of nitrogen, fission yeast arrests the cell cycle in G1, a prerequisite for mating and meiosis. Nitrogen starvation inhibits the TORC1 activity, and mutational inactivation of TORC1 provokes phenotypes similar to those of nitrogen-starved cells, including G1 arrest and sexual development. To examine whether the TORC2 activity is also regulated in response to nitrogen availability, exponentially growing wild-type cells in the synthetic EMM medium were shifted to the same medium lacking the nitrogen source ammonium chloride (EMM-N). As reported previously, the TORC1-dependent phosphorylation of the S6 kinase Psk1 diminished and became undetectable within 30 min (Fig. 1A). In contrast, the TORC2-dependent phosphorylation of Gad8-Ser546 within the hydrophobic motif was detected along the time course of the experiment, and the phosphorylation rose to somewhat higher levels after 60 min. For up to 12 hours after shifting to the EMM-N medium, during which most of wild-type cells arrest in G1, the hydrophobic motif of Gad8 remained phosphorylated (Fig. 1B). The proteolytic processing of the GFP-Atg8 fusion protein, an autophagic marker, also confirmed inactivation of TORC1 during the experiment (Fig. 1B, bottom).

These results strongly suggest that TORC2 remains active in fission yeast cells starved of nitrogen, consistent with the observation that the activity of TORC2 and Gad8 is essential for the cell cycle arrest and sexual development after nitrogen starvation.

**The TORC2-Gad8 pathway is responsive to glucose**

It has been reported that the mitotic control under low glucose conditions is altered in a strain carrying the tor1-L2045D mutation of the TORC2 catalytic subunit. Therefore, we examined whether TORC2 activity is responsive to glucose, by shifting exponentially growing cells from the growth medium containing 3% glucose to the same medium lacking glucose. Within 5 min, Gad8 with slower electrophoretic mobility, which reflects the TORC2-dependent phosphorylation of the hydrophobic motif, became undetectable (Fig. 2A). Consistently, the in vitro kinase assay confirmed significant reduction of the Gad8 activity after glucose starvation (Fig. 2A). Furthermore, adding glucose back to the glucose-starved culture restored the Gad8 phosphorylation and catalytic activity (Fig. 2A, "+G"). Immunoblotting with anti-phospho-Ser546 antibodies confirmed the glucose regulation of the Gad8 phosphorylation in the hydrophobic motif (Fig. 2B). Indeed, phosphorylation of the hydrophobic motif is crucial to glucose-induced activation of Gad8, and alanine-substitution of Ser546 largely abrogated Gad8 activity under glucose replete conditions (Fig. S1).

The standard yeast growth media contain 2~3% glucose, but the doubling time of fission yeast is not significantly different in growth media with only 0.2% (11.1 mM) glucose; however, when the glucose concentration is reduced to 0.02%, most cells...
are starved and stop dividing with high viability. Consistently, when cells grown in the presence of 3% glucose were transferred to the same medium with 0.2% glucose, the Gad8 phosphorylation was unaltered (Fig. 2C). On the other hand, Gad8 phosphorylation disappeared within 5 min when cells were transferred to the 0.02% glucose medium. Thus, the TORC2 activity responsible for the Gad8-S546 phosphorylation appears to be responsive to glucose concentrations that affect cell physiology.

2-deoxy-D-glucose (2-DG) is imported into the cell via glucose transporters and phosphorylated by hexokinase, without undergoing further glycolysis. In contrast to 0.2% glucose, which induced the Gad8-S546 phosphorylation in glucose-starved cells, the same concentration of 2-DG failed to recover the Gad8 phosphorylation at all (Fig. 2D). Thus, it appears that glucose needs to be metabolized in the cell to stimulate the TORC2 pathway.

**Glucose-regulation of the Ryh1 GTPase contributes to TORC2 activation**

How do glucose signals control TORC2 activity? It has been reported that, in cultured human cell lines, the integrity of mTOR complexes is severely impaired after glucose deprivation due to destabilization of the SIN1 and Rictor proteins. In glucose-starved *S. pombe* cells, we found that the abundance of Sin1 and the Rictor ortholog Ste20 did not fluctuate significantly (Fig. S2). Affinity purification of TORC2 from the cell lysate before and after glucose starvation showed no striking change in the interaction between Tor1 and other TORC2 subunits (Figs. 3A, B), except a modest, gradual decrease of Sin1 (Fig. S2).

In fission yeast, extracellular glucose activates the cAMP-PKA signaling pathway, of which constituents include the glucose-sensor Git3 GPCR, the Gpa2 subunit of the heterotrimeric G protein, the adenylate cyclase Cyr1, the cAMP phosphodiesterase Cgs2 and the cAMP-dependent Pka1 kinase. We found that the TORC2-dependent phosphorylation of Gad8-Ser546 is comparable between the wild-type and any of the strains lacking these components of the cAMP-PKA pathway (Fig. 3C). Furthermore, as shown in Figure 3D, the Gad8 phosphorylation was not induced in glucose-starved cells after being treated with 5 mM cAMP, which is known to activate PKA in *S. pombe*. It is therefore unlikely that the cAMP-PKA pathway mediates glucose signals to control the TORC2 activity.

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**Figure 2.** Gad8 is immediately dephosphorylated upon glucose depletion in the growth medium. (A) A gad8:6HA strain (CA4770) was cultured in YES at 30°C to the early log phase and harvested at the indicated minutes after glucose depletion from the growth medium. The sample at 0 minute was taken just prior to glucose depletion. The culture was split into 2 at 20 minutes and glucose was added at the final concentration of 3% to one of the split cultures (labeled with “C”). Crude cell lysate was prepared with TCA and analyzed by anti-HA immunoblotting to detect slower migrating, phosphorylated Gad8 (P-Gad8). Relative radioactivity in the assay was indicated as Gad8 kinase activity with the activity at 0 minute fit to 100%. A wild-type strain that expresses untagged Gad8 (CA101) was used as a negative control in the assay (the rightmost lane labeled with “N”). (B) A wild type strain (CA101) grown in YES was starved of glucose. Twenty minutes after glucose depletion, glucose was added back to an aliquot of the culture and incubated for another 10 minutes (labeled with “C”). Crude cell lysate was prepared with TCA and Gad8 phosphorylation was analyzed as in Figure 1A. (C) A wild type strain (CA101) cultured in regular EMM containing 2% glucose was harvested after shifting to EMM containing the indicated concentrations of glucose. Gad8 phosphorylation was analyzed as in Figure 1A. (D) A wild type strain (972) was cultured in YES and starved of glucose for 20 minutes, followed by 5 minutes incubation in the absence (“−”) or in the presence (“+G”) of 0.2% glucose or in the presence of 0.2% 2-deoxyglucose (“+2DG”). Gad8 phosphorylation were indicated below the “P-Gad8” panel with the level at 0 minute fit to 100%.
The Ryh1 small GTPase is an upstream regulator of the TORC2-Gad8 pathway and its GTP-bound form is active in stimulating TORC2. To determine whether Ryh1 is involved in glucose signaling, we developed a pull-down assay to monitor activation of Ryh1, an ortholog of human Rab6. Bicadual D2 (BICD2) is an effector of human Rab6, and we found that a 109-amino acid fragment of BICD2 immobilized on agarose beads can precipitate GTP-bound, active Ryh1Q70L, but not the GDP-locked Ryh1T25N mutant (Fig. 4A, lanes 1 and 2). When cells expressing wild-type Ryh1 were transferred to the glucose-free medium, the active form of Ryh1 significantly decreased in 5 min, along with a drop in Gad8 phosphorylation (Fig. 4A, lanes 3 and 4). On the other hand, in a strain expressing GTP-locked Ryh1Q70L, Gad8 remained phosphorylated even after glucose depletion, indicating that active Ryh1 is sufficient to maintain the TORC2 activity in the absence of glucose stimuli (Fig. 4B). Together, these results suggest that the nucleotide-binding state, and therefore the activity, of the Ryh1 GTPase are responsive to extracellular glucose and that swift inactivation of Ryh1 upon glucose starvation turns off the TORC2 activity to phosphorylate Gad8.

Figure 3. The TORC2 integrity is unaltered on glucose depletion and Gad8 phosphorylation is unaffected by loss of the glucose-responsive cAMP-PKA pathway. (A) FLAG:tor1 (CA6530), FLAG:tor1 sin1:myc (CA7092), FLAG:tor1 ste20:myc (CA7087), and FLAG:tor1 wat1:myc (CA7213) strains cultured in YES were harvested before (“+”) and 5 minutes after (“–”) glucose depletion. Anti-myc immunoprecipitation was performed with non-denaturing crude cell lysate, followed by anti-FLAG and anti-myc immunoblotting to detect FLAG-Tor1 and the myc tagged TORC2 components, respectively. Gad8 phosphorylation was analyzed as in Figure 1A in crude cell lysate prepared with TCA. (B) bit61:myc (CA6859) and bit61:myc FLAG:tor1 (CA6855) strains were cultured in YES and treated for glucose starvation for 5 minutes. Anti-FLAG immunoprecipitates and input were analyzed as in Figure 3A. (C) Wild-type (CA3), Δgpa2 (CA5657), Δgpa3 (CA5657), Δgpa4 (CA5657), Δgpa5 (CA5657), Δgpa6 (CA5657), Δgpa7 (CA5657), Δgpa8 (CA5657), and Δgad8 (CA142) strains grown in YES were examined for Gad8 phosphorylation as in Figure 1A. (D) A wild-type strain (CA142) was cultured in YES and starved of glucose for 20 minutes, followed by 5 minutes incubation in the presence of 0.2% glucose or 5 mM cAMP. Gad8 phosphorylation was analyzed as in Figure 1A.

Figure 4. Ryh1 responds to glucose and promotes Gad8 phosphorylation by TORC2. (A) Fission yeast strains expressing FLAG-Ryh1 of wild type ("WT," CA6809), the GTP-locked Q70L mutant (CA6817), or the GDP-locked T25N mutant (CA6828) were cultured in YES (Glucose "+"). Anti-FLAG immunoprecipitation was performed with non-denaturing crude cell lysate, followed by anti-FLAG immunoblotting to detect FLAG-Ryh1 and the myc tagged TORC2 components. Gad8 phosphorylation was analyzed as in Figure 1A. (B) A wild type ("WT," CA6809) and a GTP-locked Q70L mutant (CA6817) FLAG-ryh1 strains were cultured in EMM and harvested with glucose depletion treatment. Gad8 phosphorylation was analyzed as in Figure 1A.
Ryh1-dependent and -independent mechanisms for TORC2 activation

In order to evaluate the contribution of the Ryh1 GTPase to the glucose regulation of TORC2, we compared the TORC2-dependent phosphorylation of Gad8 in wild-type and *ryh1* null (*Δryh1*) mutant cells before and after glucose starvation. In the presence of glucose, Gad8 was significantly less phosphorylated in *Δryh1* than in wild-type cells as reported previously (Fig. 5A, time 0), confirming the role of Ryh1 as a TORC2 activator. Within 5 min after shifted to the glucose-free medium, wild-type cells showed significant reduction in Gad8 phosphorylation, due to the inactivation of Ryh1; unexpectedly, however, the phosphorylation gradually recovered to the initial level by the 30-min time point. Also in *Δryh1* cells, the Gad8 phosphorylation was induced after glucose starvation to a level comparable to that in wild-type cells grown in the presence of glucose (Fig. 5A). Thus, the induced activation of the TORC2-Gad8 pathway after glucose starvation is likely to be independent of the Ryh1 GTPase. Importantly, adding glucose back to the starved *Δryh1* cells brought down the Gad8 phosphorylation to the initial level in the presence of glucose (Fig. 5A, "+G"), implying that the putative Ryh1-independent activator of TORC2 is negatively regulated by glucose. Similar results were obtained also with the GDP-locked *ryh1T25N* mutant (Fig. S3).

Collectively, when the growth medium is replete with glucose, the GTP-bound, active form of Ryh1 promotes TORC2-Gad8 signaling, while meager supplies of glucose inhibit Ryh1, resulting in reduced TORC2 activity. On the other hand, glucose starvation triggers a Ryh1-independent mechanism that can fully reactivate the TORC2-Gad8 pathway.

Wild-type fission yeast cells can proliferate even when the glucose concentration in the growth medium is lowered to \(0.08 \sim 0.2\%\).\(^8\) We noticed, however, that the *Δtor1* and *Δgad8* mutant strains failed to grow under such glucose-limited conditions (Fig. 5B), indicating that active TORC2-Gad8 signaling is essential for cellular response to glucose limitation. In contrast, cells carrying an inactive allele of *ryh1* proliferated in both high and low glucose media (Fig. 5C), consistent with the idea that the Ryh1-independent activation of the TORC2-Gad8 pathway allows cell division under glucose limitation.

**Discussion**

In this study, we have demonstrated that the fission yeast TORC2-Gad8 pathway is responsive to glucose and plays an essential role in cell proliferation under glucose-limited conditions. Intriguingly, the homologous mTORC2-Akt pathway in...
animals regulates the cellular uptake and metabolism of glucose in response to insulin. It is tempting to speculate that insulin signaling in multicellular organisms may have evolved from a glucose-responsive pathway in unicellular eukaryotes. On the other hand, TORC1 in S. pombe is stimulated by nitrogen sources such as ammonium, and its mutational inactivation induces cellular responses similar to those triggered by nitrogen starvation. Thus, the TOR signaling pathways in fission yeast seem to mediate major nutritional input, nitrogen and carbon/energy.

The molecular mechanism that activates mTORC2 in the insulin signaling pathway remains elusive, though involvement of lipid second messengers and ribosome has been suggested. In S. pombe, a genetic screen identified the Rab-family GTPase Ryh1 and its guanine nucleotide exchange factor (GEF) as positive regulators of TORC2. We have further extended this observation by showing in this paper that the nucleotide-binding state of Ryh1 is regulated in response to glucose. Upon glucose starvation, the GTP-bound, active form of Ryh1 significantly decreases, resulting in diminished TORC2 activity. Furthermore, the TORC2-Gad8 pathway is insensitive to glucose starvation in cells expressing the constitutively active Ryh1Q70L protein, strongly suggesting that the glucose-dependent regulation of Ryh1 controls TORC2 activity.

Comparing to other protein kinases, mTOR requires higher levels of ATP for its catalytic activity. In cultured human cells, glucose starvation brings about a gradual decrease of cellular ATP to less than 15% of the normal level, resulting in destabilization of the essential mTORC2 subunits SIN1 and Rictor. In contrast, we found that glucose deprivation does not significantly affect the abundance of the TORC2 subunits in fission yeast. The cellular ATP level in fission yeast goes down only to ~80% at 30 min after a shift from 2% to 0.02% glucose, and a treatment that markedly reduces Gad8 phosphorylation by TORC2 within 5 min (Fig. 2C). Moreover, in cells expressing the Ryh1Q70L mutant protein, TORC2 remains active even after glucose starvation. Thus, reduced cellular ATP levels do not seem to be responsible for the swift inactivation of TORC2 in fission yeast cells deprived of glucose.

Cohen et al. recently reported that glucose signals are mediated via cAMP-PKA to activate the TORC2-Gad8 pathway, because the Gad8-Ser546 phosphorylation was not detectable in the Δgit3, Δgpa2 and Δpka1 mutants even under glucose-replete conditions. In contrast, our repeated experiments detected no significant defect in the Gad8 phosphorylation among the cAMP-PKA pathway mutants tested in this study, including Δgit3, Δgpa2 and Δpka1. Because Cohen et al. prepared the cellular protein extract under non-denaturing condition without phosphatase inhibitor, it is conceivable that Gad8 was dephosphorylated by phosphatases in the crude extract. They also used strains expressing Gad8 with a C-terminal epitope tag, which could affect their experiment to monitor phosphorylation of the hydrophobic motif close to the C-terminus. In our experiments (Fig. 3C), we examined the phosphorylation state of the endogenous Gad8 protein with no tagging. In addition, the lysate was prepared from TCA-fixed cells under denaturing condition in order to prevent protein phosphorylation/dephosphorylation after cell disruption. It should also be noted that not only cAMP but also the glucose analog 2-DG fails to activate the TORC2 pathway; therefore, glucose needs to be metabolized in the cell to stimulate TORC2, rather than being sensed by the cell-surface GPCR upstream of the cAMP-PKA pathway.

Despite the notable role of the Ryh1 GTPase in mediating glucose-induced activation of TORC2, strains without functional Ryh1 are not sensitive to low-glucose conditions. Indeed, glucose deprivation induces activation of the TORC2-Gad8 pathway in the absence of Ryh1 and therefore, the ryh1-defective cells have enough TORC2-Gad8 activity to grow with limited glucose. In wild-type cells, the TORC2 pathway is inhibited immediately after glucose starvation due to the prompt inactivation of Ryh1, but subsequently, the pathway becomes reactivated. Comparing to the Δryh1 mutant, the reactivation is slower in the wild-type strain, possibly due to the negative feedback regulation, in which the initial high activity of Gad8 could delay the TORC2 reactivation. Currently, the mechanism for the observed Ryh1-independent activation of the TORC2-Gad8 pathway is unknown. This novel mechanism appears to be inhibited by glucose, because it is operative only after glucose starvation and, in the absence of Ryh1, added glucose immediately abrogates TORC2-Gad8 signaling induced after glucose starvation.

In summary, our study has revealed the 2 regulatory mechanisms that control the TORC2-Gad8 pathway, which is essential for cellular glucose response. One is the Ryh1 GTPase whose GTP-bound form activates the TORC2 pathway under glucose-rich conditions. The other stimulates TORC2-Gad8 signaling independently of Ryh1 and is negatively regulated by glucose. The pair of these positive and negative regulatory mechanisms for TORC2 may allow fine-tuning of the pathway to optimize the cellular growth under changing glucose conditions. The exact function of the TORC2-Gad8 pathway remains to be elucidated. In addition, the TORC2-Gad8 pathway in cellular response to glucose has to be determined. Like mTORC2-Akt, the fission yeast TORC2 pathway may be involved in the uptake and/or metabolism of glucose. It has also been found that TORC2 is required to modulate the mitotic cell cycle in response to low glucose conditions. We expect that further investigation of glucose signaling in fission yeast will contribute to the discovery of evolutionarily conserved regulation and function of TORC2 signaling.

Materials and Methods

Fission yeast strains and general techniques
Fission yeast strains used in this study are listed in Table 1. Standard growth media, basic techniques, and genetic manipulations of fission yeast have been described. Crude cell lysate preparation using trichloroacetic acid (TCA) has been reported. The Gad8 in vitro kinase assay has been described previously. Immunoprecipitation was performed as described before. Luminescent Image Analyzer LAS-4000 (Fujifilm) was used for signal quantification in immunoblotting. For nitrogen starvation,
cells grown in liquid EMM were filter-harvested and transferred into pre-warmed fresh EMM lacking NH₄Cl. For glucose depletion, cells grown in standard YES or EMM were filter-harvested and transferred to glucose free YES or EMM, in which sorbitol was added to maintain medium osmolarity after glucose depletion, except for Figures 2A, 2D, S1 and S2. For glucose add-back, cells starved of glucose for 20 minutes were filter-harvested and introduced in glucose rich media, followed by 5 or 10 minutes incubation.

Detection of autophagy and Psk1 phosphorylation

Autophagy was monitored as described previously. Briefly, the GFP-Atg8 fusion protein, which was expressed exogenously under the control of the thiamine-repressible nmt1 promoter of pREP4166 in the absence of thiamine, was examined in immuno-blotting with antibodies against GFP for its autophagy-induced cleavage. Psk1 phosphorylation levels were analyzed as described before.  

Ryh1 pull-down assay

The C-terminal 706 – 814th amino acid region of human Bicaudal D2 (BICD2) was fused with glutathione S-transferase (GST) and expressed in the E. coli BL21 strain at 16°C. The fusion protein was purified by glutathione Sepharose chromatography (GE Lifesciences) in lysis buffer (25 mM Tris-HCl [pH 7.5], 137 mM NaCl, 2.7 mM KCl, 1% triton X-100, and 1 mM PMSF). Yeast cells expressing the FLAG epitope tagged Ryh1 were cultured in YES liquid media at 30°C and harvested by filtration. Harvested cells were washed once with ice-cold water and stored at −80°C until use.

Yeast cells were lysed in ice-cold PBS containing 10 mM MgCl₂, 0.5% Tween 20, 1 mM PMSF, and the protease inhibitor cocktail for use in purification of histidine-tagged proteins (Sigma #P8849). Crude cell lystate was cleared by centrifugation (GE Lifesciences) in lysis buffer (25 mM Tris-HCl [pH 7.5], 137 mM NaCl, 2.7 mM KCl, 1% triton X-100, and 1 mM PMSF). Yeast cells expressing the FLAG epitope tagged Ryh1 were cultured in YES liquid media at 30°C and harvested by filtration. Harvested cells were washed once with ice-cold water and stored at −80°C until use.

No potential conflicts of interest were disclosed.

Table 1. Fission yeast strains used in this study

| strain ID | genotype | reference/source |
|----------|----------|------------------|
| 972      | h        |                  |
| CA101    | h leu1–32|                  |
| CA3      | h leu1-32 ura4-D18 | Laboratory stock |
| CA4593   | h leu1–32 ura4-D18 tor1::ura4+ | Laboratory stock |
| CA4636   | h leu1–32 ura4-D18 ade6-M210 gad8::ura4+ gad8T387A::6HA(kanMX6) | This study |
| CA4770   | h leu1–32 ura4-D18 gad8::ura4+ gad8T387A::6HA(kanMX6) | Laboratory stock |
| CA4968   | h leu1–32 ura4-D18 sin1::FLAG(kanMX6) ste20::myc(kanMX6) | This study |
| CA5019   | h leu1–32 ura4-D18 wat1::FLAG(kanMX6) | This study |
| CA5142   | h leu1–32 ura4-D18 gad8::ura4+ | Laboratory stock |
| CA5256   | h ura4-D18 ura4-D18 gad8::ura4+ << karr gad8::ura4+ | This study |
| CA5473   | h leu1–32 ura4-D18 sin1::FLAG(kanMX6) ste20::myc(kanMX6) NTAP:tor1 | This study |
| CA5484   | h leu1–32 ura4-D18 wat1::FLAG(kanMX6) NTAP:tor1 | This study |
| CA5512   | h ura4-D18 git3::ura4+ | This study |
| CA5513   | h ura4-D18 gpa2:ura4+ | This study |
| CA5603   | h leu1–32 ura4-D18 pka1::ura4+ | This study |
| CA5604   | h leu1–32 ura4-D18 ade6-M216 cyr1::ura4+ | This study |
| CA5605   | h leu1–32 ura4-D18 cgs2::ura4+ | This study |
| CA5647   | h leu1–32 ura4-D18 cyr1::ura4+ | This study |
| CA5657   | h leu1–32 ura4-D18 git3::ura4+ | This study |
| CA5669   | h leu1–32 ura4-D18 gpa2::ura4+ | This study |
| CA5724   | h ura4-D18 gad8::ura4+ gad8T387A::6HA (kanMX6) | This study |
| CA6217   | h leu1–32 ryh1::kanMX6 | This study |
| CA6530   | h leu1–32 (hph)FLAG:tor1 | This study |
| CA6809   | h leu1–32 3FLAG:ryh1 | This study |
| CA6817   | h leu1–32 3FLAG:ryh1 NTAP:tor1 | This study |
| CA6828   | h leu1–32 3FLAG:ryh1T25N | This study |
| CA6855   | h leu1–32 (hph)FLAG:tor1 bit61:13myc (kanMX6) | This study |
| CA6859   | h leu1–32 bit61:13myc (kanMX6) | This study |
| CA7092   | h leu1–32 (hph)FLAG:tor1 bit61:13myc (kanMX6) | This study |
| CA7213   | h leu1–32 (hph)FLAG:tor1 wat1::13myc (kanMX6) | This study |
| CA7366   | h leu1–32 ura4-D18 3FLAG:ryh1 gad8::ura4+ | This study |
| JX105    | h ura4-D18 pka1::ura4+ | This study |
| JZ666    | h leu1–32 ura4-D18 ade6-M216 cgs2::ura4+ | This study |

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.
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