

The geranylgeranyltransferase Cwg2-Rho4/Rho5 module is implicated in the Pmk1 MAP kinase-mediated cell wall integrity pathway in fission yeast

Akira Doi^{1,2}, Ayako Kita¹, Yuki Kanda¹, Takaya Uno¹, Keita Asami¹, Ryosuke Satoh¹, Kentaro Nakano³, and Reiko Sugiura^{1*}

1 Laboratory of Molecular Pharmacogenomics, School of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577-8502, Japan.

2 Japan Society for the Promotion of Science, 1-8 Chiyoda-ku, Tokyo 102-8472, Japan

3 Department of Biological Sciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennohdai, Tsukuba, Ibaraki, 305-8577, Japan.

*To whom correspondence should be addressed: Laboratory of Molecular Pharmacogenomics, School of Pharmaceutical Sciences, Kinki University, Higashi-Osaka 577-8502, Japan.

TEL: +81-6-6730-5880 (ext. 3850), FAX: +81-6-6730-1394, E-MAIL: sugiurar@phar.kindai.ac.jp

Short title: Cwg2/Rho and MAPK in *S. pombe*

Key words: MAP kinase, geranylgeranyltransferase, Rho, protein kinase C, fission yeast.

Abstract

Pmk1, a fission yeast homologue of mammalian ERK MAPK, regulates cell wall integrity, cytokinesis, RNA granule formation and ion homeostasis. Our screen for *vic* (viable in the presence of immunosuppressant and chloride ion) mutants identified regulators of the Pmk1 MAPK signaling, including Cpp1 and Rho2, based on the genetic interaction between calcineurin and Pmk1 MAPK. Here, we identified the *vic2-1* mutants carrying a missense mutation in the *cwg2⁺* gene encoding a beta subunit of geranylgeranyl transferase I (GGTase I), which participates in the post-translational C-terminal modification of several small GTPases, allowing their targeting to the membrane. Analysis of the *vic2-1/cwg2-v2* mutant strain revealed that the localization of Rho1, Rho4, Rho5 and Cdc42, both at the plasma and vacuolar membranes was impaired in the *vic2-1/cwg2-v2* mutant cells. In addition, Rho4 and Rho5 deletion cells exhibited the *vic* phenotype and cell wall integrity defects, shared phenotypes among the components of the Pmk1 MAPK pathway. Consistently, the phosphorylation of Pmk1 MAPK upon heat shock was decreased in the *cwg2-v2* mutants, and *rho4-* and *rho5-*null cells. Moreover, Rho4 and Rho5 associate with Pck1/Pck2. Possible roles of Cwg2, Rho4 and Rho5 in the Pmk1 signaling will be discussed.

Introduction

The mitogen-activated protein kinase (MAPK) pathway is one of the most important intracellular signaling pathways that transduce signals from the cell membrane to the nucleus and play a crucial role in various cellular processes, including cell proliferation, cell differentiation, stress responses and cell cycle regulation (Nishida & Gotoh 1993; Marshall 1994; Herskowitz 1995; Levin & Errede 1995; Munshi & Ramesh 2013).

The Pmk1 MAPK, a fission yeast homologue of the mammalian ERK/MAPK, regulates cell morphology, cell wall integrity, cytokinesis, ion homeostasis, genotoxic stress and stress granule formation in the fission yeast *Schizosaccharomyces pombe* (Toda *et al.* 1996; Zaitsevskaya-Carter & Cooper 1997; Sugiura *et al.* 1998; Broadus & Gould 2012; Satoh *et al.* 2012). The Pmk1 MAPK pathway consists of a three-tiered cascade composed of a MAPK kinase kinase (MAPKKK; Mkh1), a MAPK kinase (MAPKK; Pek1) and Pmk1 MAPK. The small GTPases Rho2 and Rho1 act upstream of the Pmk1 MAPK pathway by transmitting the signal through the protein kinase C homologue Pck1/Pck2 (Arellano *et al.* 1999). A recent study by Sanchez-Mir *et al.* reported that Rho1 and Rho2 control Pmk1 basal activity during vegetative growth mainly through Pck2, whereas Rho2-Pck2 elicit Pmk1 activation in response to most environmental stimuli, Rho1 drives Pmk1 activation through either Pck2 or Pck1 exclusively in response to cell wall damage (Toda *et al.* 1996; Sengar *et al.* 1997; Arellano *et al.* 1999; Calonge *et al.* 2000; Sanchez-Mir *et al.* 2014). The fission yeast genome contains six Rho-type small GTPases, Rho1, Rho2, Rho3, Rho4, Rho5 and Cdc42. It has been reported that Cdc42 GTPase does not act upstream of Pmk1 signaling based on the finding that Cdc42 does not control the basal level or the stress-induced activation of Pmk1 (Madrid *et al.* 2006). However, little is known about the functional connections between other Rho-family members, such as Rho4/Rho5 and Pmk1 MAPK signaling in fission yeast.

We have previously demonstrated that knockout of the fission yeast calcineurin gene *ppb1*⁺ or inhibition of calcineurin activity by the immunosuppressant FK506 results in hypersensitivity to Cl⁻, and that calcineurin and Pmk1 MAPK play antagonistic roles in Cl⁻ homeostasis (Sugiura *et al.* 1998). Based on this genetic interaction between calcineurin and Pmk1 MAPK, we established a genetic screen to isolate *vic* (viable in the presence of immunosuppressant and chloride ion) mutants, aiming to identify novel components of the Pmk1 MAPK pathway. The genes we identified include a mutation in the *cpl1*⁺ gene, encoding a beta subunit of the farnesyltransferase (FTase). Cpl1 was shown to act upstream of the Pck2-Pmk1 MAPK cell wall integrity signaling pathway by regulating Rho2 localization (Ma *et al.* 2006). In addition, deletion of the Atf1 transcription factor, which was shown to be phosphorylated by Pmk1 in response to cell wall perturbation, also induced the *vic* phenotype and micafungin sensitivity (Takada *et al.* 2007), thus showing that the *vic* phenotype was effective in isolating molecules acting both upstream and downstream of the Pmk1 MAPK pathway.

Using the screen for *vic* mutants, here we identified the *cwg2-v2* mutation which caused an amino acid substitution of arginine 254 of Cwg2 to lysine (Cwg2^{R254K}) as a *vic2-1* mutant. The *Schizosaccharomyces pombe* *cwg2*⁺ gene encodes the beta-subunit of a geranylgeranyl transferase I (GGTase I), which participates in the post-translational C-terminal modification of several small GTPases, allowing their targeting to the membrane (Ribas *et al.* 1991; Diaz *et al.* 1993; Arellano *et al.* 1996; Arellano *et al.* 1998). Cwg2 is known to be required for β -glucan synthesis and cell morphology by functionally interacting with Rho1 (Arellano *et al.* 1996; Arellano *et al.* 1998). Here, we show that the *vic2-1/cwg2-v2* mutation affects the intracellular localization of several small GTPases, including Rho1, Rho4, Rho5 and Cdc42, which resulted in dysregulation of subsequent activation of Pmk1 mediated signaling. We also showed that in addition to Rho1, which is the previously well-characterized

activator of the Pmk1 pathway, Rho4 and Rho5 are implicated in the Pmk1-mediated cell wall integrity signaling pathway. The possible roles of Cwg2, Rho4 and Rho5 in the Pmk1-mediated cell wall integrity signaling will be discussed.

Results

The *vic2-1/cwg2-v2* is an allele of the *cwg2*⁺ gene that encodes a beta subunit of the protein GGTase I

To identify new components of the Pmk1 MAPK signaling pathway, we screened for mutants which exhibited the *vic* phenotype in *S. pombe*. As shown in Fig. 1A, the *vic2-1* mutants grew well in the presence of FK506 and 0.15 M MgCl₂ at 27°C whereas the wild-type cells failed to grow (Fig. 1A; EMM +FK506+0.15 M MgCl₂). Moreover, *vic2-1* mutant cells could not grow at 36°C whereas the wild-type (wt) cells normally grew (Fig. 1A; YPD 36°C). The *vic2*⁺ gene was cloned by complementation of the temperature-sensitive growth defect of *vic2-1* mutant cells (Fig. 1A; YPD 36°C, *vic2-1+cwg2*⁺). Nucleotide sequencing of the cloned DNA fragment revealed that the *vic2*⁺ gene is identical to the *cwg2*⁺ gene (SPAC2E1P5.04c), which encodes the beta subunit of the protein GGTase I of 355 amino acids that are highly similar to the human GGTase PGGT1B and the mouse GGTase Pgg1b (Fig.1B alignment) (Arellano *et al.* 1998). Linkage analysis was performed (see MATERIALS AND METHODS) and results indicated the allelism between the *cwg2*⁺ gene and the *vic2-1* mutation. We therefore renamed *vic2-1* as *cwg2-v2*. The *vic2*⁺ gene also complemented the *vic* phenotype and the temperature sensitivity of *vic2-1* mutant cells, as these cells grew well at 36°C and failed to grow in the presence of FK506 and 0.15 M MgCl₂, when the *cwg2*⁺ gene was introduced (Fig. 1A; *vic2-1/cwg2-v2+cwg2*⁺).

We then identified the mutation site in the *cwg2-v2* allele, by isolating the genomic DNA from the *cwg2-v2* mutant cells and sequencing the full-length coding region of the *cwg2-v2* gene. The G to A nucleotide substitution caused a highly conserved arginine to be altered to a lysine residue at the amino acid position 254 (Fig. 1B; black arrow). This arginine residue was located in the catalytic domain of GGTase I, and thus may be important for the GGTase I activity. We therefore refer to the protein product of the *cwg2-v2* gene as Cwg2^{R254K}.

In addition, the genetic interaction between *cwg2-v2* and Δ *pmk1* was examined by constructing *cwg2-v2* Δ *pmk1* double mutant cells. The degree of the *vic* phenotype in *cwg2-v2*, Δ *pmk1*, and *cwg2-v2* Δ *pmk1* double mutant was almost equivalent, as they grew in the presence of 0.1 M MgCl₂ and FK506 (Fig. 1C). In addition, the *cwg2-v2*, Δ *pmk1*, and *cwg2-v2* Δ *pmk1* double mutant failed to grow in the presence of 1.0 μ g/ml micafungin, indicating that *cwg2-v2* mutants exhibited cell wall integrity defects, a shared phenotype of the Pmk1 MAPK signaling pathway (Fig. 1C). Cell wall integrity defect of these mutants was further examined using a cell wall-digesting enzyme β -glucanase, and the results confirmed that the kinetics of cell lysis in the *cwg2-v2* Δ *pmk1* double mutant was almost similar to that in Δ *pmk1*, indicating that *cwg2-v2* is epistatic to Δ *pmk1* (Fig. 1D). Because a previous paper on Cwg2 reported that the *cwg2*-null mutants were inviable, and exhibited aberrant morphology and septation defects (Arellano *et al.* 1998), we examined the morphological and cytokinesis abnormalities in the *cwg2-v2* mutant cells. Contrary to expectation, the *cwg2-v2* mutants showed no striking abnormalities at the permissive temperature of 27°C (Fig. 1E, *cwg2-v2*). We then analyzed the septation defects in the *cwg2-v2*, Δ *pmk1*, and *cwg2-v2* Δ *pmk1* double mutant, and the *cwg2-v2* Δ *pmk1* double mutants exhibited a markedly severer septation defects than each parental single mutants at the permissive temperature, regarding both the septation index and the frequency of multi-septated cells (Fig. 1E). Thus, the *cwg2-v2* and the Δ *pmk1* were additive in terms of the morphological and cytokinesis regulation pathway, indicating that Cwg2 and Pmk1 act synergistically in these functions.

The *cwg2-v2* mutation affects the intracellular localization of multiple Rho GTPases

GGTase I catalyzes the post-translational modification of target proteins including Rho-family proteins (Lane & Beese 2006). The target proteins have a consensus CAAX motif (where C represents cysteine, A represents aliphatic amino acid, and X

preferentially represents leucine or phenylalanine) at the C-terminus (Lane & Beese 2006). Because our intention of isolating *vic* mutants is to identify new components that act in the Pmk1 MAPK pathway, we assumed that one of the attractive candidate targets of Cwg2 responsible for the *vic* phenotype upon mutation would be Rho-family members based on the conserved roles of Rho proteins as upstream activators of the MAPK signaling (Takai *et al.* 2001). In fission yeast, among the six Rho-family proteins, Rho1, Rho4, Rho5, and Cdc42 small GTPases contain CAAL motif modified by GGTase I (Fig. 2A). Because prenylation dictates the membrane association of target proteins, we explored whether the *cwg2-v2* mutation would affect localization of the target Rho proteins. To answer this question, the intracellular localization of GFP-fused Rho1, Rho4, Rho5, and Cdc42 in wild-type (wt) or *cwg2-v2* mutant cells was examined. As shown in Fig. 2B, in wt cells all the examined GFP-fused Rho proteins predominantly localized to the plasma membrane with strong signals at the cell ends (Fig. 2B; wt, arrows). On the other hand, in mitotic cells, the GFP fluorescence of these small GTPases was observed in the medial region of the cell (Fig. 2B; wt, arrowheads). In contrast, in *cwg2-v2* mutant cells, the levels of these Rho proteins at the plasma membrane were significantly lower than in wt cells at the permissive temperature (Fig. 2B; *cwg2-v2*). Notably, GFP-fused Rho5 and Cdc42 at the plasma membrane were hardly detectable in *cwg2-v2* mutant cells (Fig. 2B; *cwg2-v2*). The fluorescence of these GFP-Rho proteins in the medial region of the cell was also weakened in *cwg2-v2* mutant cells (Fig. 2B; *cwg2-v2*).

Notably, the fluorescence of GFP-fused Rho1, Rho4, Rho5 and Cdc42 proteins was also visible in the endomembranes (Fig. 2C; wt, double arrowheads). We therefore examined the effect of the *cwg2-v2* mutation on the endomembrane localization of these Rho proteins. For this purpose, we incubated cells expressing GFP-fused Rho proteins with FM4-64, a fluorescent dye which visualizes the endocytic pathway to the vacuole for 60 min, and then incubated the cells with water for 60 min to

induce vacuolar fusion (Kita *et al.* 2004). In wt cells, the fluorescence of FM4-64 and GFP-fused Rho1, Rho4, Rho5 and Cdc42 proteins in the internal membranes was largely co-localized, suggesting that these Rho proteins were localized to the vacuolar membranes inside the cells (Fig. 2C; wt, double arrowheads).

In contrast, in *cwg2-v2* mutant cells the fluorescence of GFP-Rho1, Rho4, Rho5 and Cdc42 in the internal membranes was markedly diminished (Fig. 2C; *cwg2-v2*, GFP). It should be noted however, that in *cwg2-v2* mutant cells expressing GFP-Rho proteins, the fluorescence of FM4-64 was clearly visible on the vacuolar membranes (Fig. 2C; *cwg2-v2*, FM4-64), although the size of vacuoles was a little smaller than those of the wt cells, thus indicating that the weakened signal of these GFP-Rho proteins was not due to the vacuolar dysfunction of the *cwg2-v2* mutant cells (Fig. 2C; *cwg2-v2*). These results suggest that the *cwg2-v2* mutation affected geranylgeranylation of multiple target Rho proteins, thereby leading to mislocalization of these Rho proteins at both the plasma and vacuolar membranes. It should be noted that the *cwg2-v2* mutation did not affect the localization of Rho2 protein, which was shown to be a target of the FTase Cpp1 (Fig. 2B, C; GFP-Rho2).

Rho4 and Rho5 participate in the Pmk1-mediated cell wall integrity signaling pathway

The aforementioned data suggested that the *vic* phenotype associated with the *cwg2-v2* mutation may be explained by the mislocalization and subsequent dysfunction of target Rho proteins which act in the Pmk1 MAPK pathway. Therefore, we explored if knockout of these Rho genes may exhibit phenotypes shared among components of the Pmk1 pathway, including the *vic* phenotype and the cell wall integrity defect. If target Rho proteins are indeed involved in the Pmk1 pathway, it would be expected that deletion of the Rho genes may induce phenotypes shared among components of the Pmk1 pathway. Among four viable Rho deletion cells (Rho2, Rho3, Rho4 and Rho5),

we have previously shown that $\Delta rho2$ cells displayed a *vic* phenotype and $\Delta rho3$ cells were categorized as non-*vic* mutants (Ma *et al.* 2006). Notably, knockout of $rho4^+$ and $rho5^+$ genes induced *vic* phenotype because Rho4 and Rho5 deletion cells were viable in the presence of FK506 and 0.1 M $MgCl_2$, whereas the wild-type cells failed to grow (Fig. 3). Because mutations that perturb the signaling through the Pmk1 MAPK pathway are known to result in defective cell wall integrity (Toda *et al.* 1996), we thus examined whether knockout of $rho4^+$ and $rho5^+$ genes display hypersensitivity to the cell wall-damaging agent micafungin, an inhibitor of (1,3)- β -D-glucan synthase (Carver 2004). Rho4 and Rho5 deletion cells exhibited micafungin sensitivity, although the degree of sensitivity was severer in $\Delta pmk1$ as compared with those of $\Delta rho4$ and $\Delta rho5$ cells (Fig. 3; YPD + 1.0 $\mu g/ml$ micafungin). The loss-of-function allele *cdc42L160S* mutant cells did not exhibit the *vic* phenotype (Fig. 3; YPD + FK506 + 0.1 M $MgCl_2$), which is in agreement with the previous reports showing that Cdc42 does not act upstream of the Pmk1 pathway (Madrid *et al.* 2006). Together, the above data suggested the possibility that in addition to Rho1, a well-characterized upstream activator of Pmk1, Rho4 and Rho5 may be involved in the regulation of the Pmk1 signaling pathway. We therefore focused on the functional analysis of Rho4 and Rho5 as to whether they act in the Pmk1 pathway.

Rho4 and Rho5 are involved in the Pmk1 MAPK pathway

As an initial step to gain insight into how Rho4 and Rho5 are involved in the Pmk1 pathway, we investigated whether Rho4 and Rho5 act upstream of the Pmk1 pathway. We then examined the effects of the mutation or deletion of *cwg2⁺*, *rho4⁺*, and *rho5⁺* genes on the phosphorylation levels of Pmk1 using anti-phospho-Pmk1 antibodies. In wt cells, Pmk1 phosphorylation was induced in response to heat shock (Fig. 4A; wt). In contrast, in *cwg2-v2* mutants and *rho4*-null cells, Pmk1 phosphorylation levels in response to heat shock were markedly decreased as compared with the wt cells (Fig. 4A). In *rho5*-null cells, Pmk1 phosphorylation was slightly, but significantly

decreased as compared with the wt cells (Fig. 4A). The quantification of Pmk1 phosphorylation normalized with anti-GST blots also confirmed the above results (Fig. 4A; lower panel).

We next examined the effect of the overexpression of *rho4*⁺ and *rho5*⁺ on the phosphorylation levels of Pmk1 in wild-type cells. Previous studies from our laboratory and others demonstrated that overexpression of *rho2*⁺ stimulated Pmk1 signaling and that of *cdc42*⁺ did not (Ma et al. 2006; Madrid et al. 2006). As shown in Fig. 4B, overexpression of *rho4*⁺ in wt cells stimulated phosphorylation of Pmk1 as compared with the control vector alone (Fig. 4B; vector, + Rho4). In addition, overexpression of *rho5*⁺ resulted in the stimulation of Pmk1 phosphorylation (Fig. 4B; + Rho5). Thus, Rho4 and Rho5 may likely act as activators of the Pmk1 signaling in the vegetative stage.

Functional interactions between Cwg2, Rho4, Rho5 and the Pmk1-mediated cell wall integrity signaling pathway

To distinguish whether or not Rho4/Rho5 proteins and Pmk1 MAPK act in a single cascade, $\Delta\rho4\Delta pmk1$ and $\Delta\rho5\Delta pmk1$ double mutants were made and phenotypes were compared with each single mutant. When we carefully examined the *vic* phenotype of these mutant cells, we noticed that the degree of the *vic* phenotype associated with *rho4* and *rho5*-null cells was intermediate between wt and $\Delta pmk1$ cells, as $\Delta\rho4$ and $\Delta\rho5$ failed to grow when higher concentrations of MgCl₂ were added with FK506 in the medium, wherein $\Delta pmk1$ grew (Fig. 5A; + FK506 + 0.12 M MgCl₂). Notably, the *vic* phenotype in $\Delta\rho4\Delta pmk1$ and $\Delta\rho5\Delta pmk1$ double mutant cells was enhanced as compared with those shown in each single mutant. As shown in Fig. 5A, in the plates containing 0.12 M MgCl₂ supplemented with FK506, the growth of $\Delta\rho4\Delta pmk1$ and $\Delta\rho5\Delta pmk1$ double mutant cells was better than those of the single $\Delta\rho4$, $\Delta\rho5$, and $\Delta pmk1$ cells.

As shown in Fig. 3, the micafungin sensitivities of $\Delta rho4$ and $\Delta rho5$ cells were intermediate between wt and $\Delta pmk1$ cells. Notably, the degree of the micafungin sensitivities of $\Delta rho4\Delta pmk1$ and $\Delta rho5\Delta pmk1$ double mutant cells was almost equivalent to that of the single $\Delta pmk1$ cells (Fig. 5A; + 1.0 $\mu\text{g/ml}$ micafungin, + 1.2 $\mu\text{g/ml}$ micafungin; Fig. 5B; + 0.3 $\mu\text{g/ml}$ micafungin), thus indicating that Rho4 and Rho5 are epistatic to Pmk1 deletion with respect to cell wall integrity defects.

To further characterize the cell wall integrity defect in these mutant cells, β -glucanase treatment was performed. As shown in Fig. 5C $rho4$ - and $rho5$ -null cells exhibited moderate sensitivities to cell wall digestion as compared with the wt cells, because these mutant cells lysed faster than wt cells upon β -glucanase treatment. Pmk1 deletion cells exhibited more severe sensitivity to β -glucanase than $rho4$ - and $rho5$ -null cells (Fig. 5C). Notably, the sensitivity of $\Delta rho4\Delta pmk1$ double mutants and $\Delta rho5\Delta pmk1$ double mutants was almost similar to that of the $\Delta pmk1$ single mutants, again confirming that Rho4 and Rho5 are epistatic to Pmk1 deletion with respect to cell wall integrity defects. These results indicate a synergism between $\Delta rho4/\Delta rho5$, and $\Delta pmk1$ with respect to the resistance to FK506 and MgCl_2 , but not to micafungin sensitivities.

Because both Rho4 and Pmk1 are involved in the regulation of cell morphology (Toda *et al.* 1996; Nakano *et al.* 2003), we investigated morphological alterations in the single and double mutant cells. As shown in Fig. 5D, both $\Delta pmk1$ and $\Delta rho4$ cells exhibited septation abnormalities; such as multi-septation phenotypes, which were negligible in the wt cells (Fig. 5D). Notably, the morphological alterations were markedly enhanced in the double $\Delta rho4\Delta pmk1$ cells as more than 30% of the cells had septation, and multi-septated cells were frequently observed (Fig. 5D). Thus, Rho4 and Pmk1 act synergistically in the regulation of morphology. In contrast to $\Delta rho4$, $\Delta rho5$ cells did not show any morphological defects *per se*, and their involvement in septation and morphology was observed only when Rho5 was

overexpressed (Nakano *et al.* 2005). Notably, however, the $\Delta rho5\Delta pmk1$ mutant cells exhibited a high septation index and multi-septated phenotypes which were exacerbated as compared with the single $\Delta pmk1$ cells, because more than around 30% of the $\Delta rho5\Delta pmk1$ cells had septation (Fig. 5D).

Physical interactions between Rho4/Rho5 and Pcks

The above results prompted us to investigate the functional relationship between Rho4/Rho5 and Pck1/2, because Pck1 and Pck2 act upstream of Mkh1 MAPKKK and transmit signaling from Rho1 and Rho2 proteins. We thus examined whether Pck1 and Pck2 associate with Rho4 or Rho5. For this, we expressed GST-fused Pck1 or Pck2 together with GFP-Rho1, Rho4, Rho5, or GFP alone. In the GST pull-down assay, GST-Pck1 associates with GFP-Rho1, GFP-Rho4, and GFP-Rho5 but not with GFP (Fig. 6A). Similarly, GST-Pck2 associates with GFP-Rho1, GFP-Rho4, and GFP-Rho5 but not with GFP (Fig. 6B). These results suggest protein-protein interactions between Pck1/2 and Rho1, Rho4 and Rho5.

Discussion

In the present study, we performed a genetic screen that aims to identify new components of Pmk1 signaling, and identified a missense mutation of the *cwg2*⁺ gene encoding a GGTase I. Characterization of the *cwg2-v2* mutation revealed a novel functional link between Rho4/Rho5 small GTPases and the Pmk1 MAPK pathway in cell wall integrity.

Cwg2 acts in a Pmk1 MAPK pathway by regulating multiple Rho GTPases

Previous information about *S. pombe* Cwg2 (GGTase I) mutants was limited to their functional interaction with Rho1 and β -D-glucan synthesis (Diaz *et al.* 1993; Arellano *et al.* 1996; Arellano *et al.* 1998). The *cwg2-v2* mutation isolated in our screen for *vic* mutants revealed its impact on multiple Rho GTPases, including Rho1, Rho4, Rho5 and Cdc42, thus the phenotypes associated with the *cwg2-v2* mutation can be partly attributable to the sum of partial loss-of-function of these Rho proteins. The phenotypes of the *cwg2-v2* mutants regarding defects in cell wall integrity as evidenced by sensitivity to micafungin and β -glucanase are consistent with previous papers reporting that Rho1, Rho4, and Rho5 are involved in septum formation and cell wall integrity, although each Rho protein participates in these functions in a different manner. Rho1 is an essential multifunctional protein that regulates actin localization, cell polarity, septum formation, and cell wall integrity acting as a regulatory subunit of (1,3)- β -D-glucan synthase (Arellano *et al.* 1997; Nakano *et al.* 1997), thus having a profound effect on cytokinesis. Rho4 is also involved in cell morphology, septation and cell wall integrity, presumably by affecting secretion of β -glucanases (Nakano *et al.* 2003; Santos *et al.* 2003; Santos *et al.* 2005). Rho5 is a functional homologue of Rho1, and involved in cell wall/septum formation, although the *rho5*-null cells did not exhibit a significant defect in cell wall formation (Nakano *et al.* 2005; Rincon *et al.* 2006). Cdc42 is involved in controlling polarized cell growth, and regulates multiple membrane traffic events, which may also lead to cell wall integrity defects upon

mutation (Estravis *et al.* 2011; Rincon *et al.* 2014). Consistently, the *cwg2-v2* mutants as a result of a combination of these Rho protein's dysfunction, displayed defects in cell wall integrity.

Because our initial aim was to identify new components which act in the Pmk1 pathway, we characterized the *cwg2-v2* mutants focusing on their relationship with Pmk1 MAPK. Among the Cwg2 target Rho proteins except for the essential Rho1 and Cdc42 small GTPases, both Rho4 and Rho5 deletion exhibited common phenotypes with *pmk1*-null, suggesting their involvement in the Pmk1 pathway. In addition, Rho4 and Rho5 act upstream of the Pmk1 pathway based on the findings that overexpression and deletion of Rho4 and Rho5 affected Pmk1 phosphorylation, and Rho4/Rho5 associated with Pck1 and Pck2, two upstream regulators of the MAPKKK Mkh1. Thus, our present study, together with previous reports from our lab and others posed several Rho GTPases as upstream regulators of the Pmk1 MAPK pathway. Which Rho proteins mainly act upstream of the Pck2-Mkh1-Pek1-Pmk1 pathway? We performed experiments to compare the impact of each Rho protein, based on its phenotypes and effects on Pmk1 phosphorylation. The severity of the micafungin sensitivity among the $\Delta rho2$, $\Delta rho4$ and $\Delta rho5$ mutant cells was compared, and the results showed that $\Delta rho2$ cells displayed the severest micafungin sensitivity, and $\Delta rho4$ and $\Delta rho5$ mutant cells exhibited relatively modest sensitivities to micafungin (Fig. S1A). In contrast, the degree of the *vic* phenotype was strongest with Rho2 deletion, and the moderate *vic* phenotypes were associated with Rho4 and Rho5 deletion (Fig. S1A). Furthermore, we compared the levels of Pmk1 phosphorylation in cells overproducing each Rho protein at a shorter induction time (20 hr after thiamine removal), and a marked increase in Pmk1 phosphorylation was observed only when the Rho2 protein was overproduced (Fig. S1B). Thus, the Cpp1/Rho2 pathway may play a major role in the activation of the Pmk1 MAPK signaling pathway.

We also performed experiments to investigate the effects of overexpression of

pck1⁺, *pck2*⁺, *rho4*⁺, and *rho5*⁺ on the micafungin sensitivity of *pmk1*-null cells (Fig. S2). The results showed that the micafungin sensitivity of *pmk1*-null cells were partially suppressed by the overexpression of *pck1*⁺, but not *pck2*⁺, *rho4*⁺, and *rho5*⁺, indicating that unlike Pck1, Rho4 and Rho5 as well as Pck2 stimulate cell integrity signaling through the Pmk1 MAPK pathway. These data, together with the epistatic analysis in Figure 5, support the hypothesis that Rho4 and Rho5 act upstream of Pck2, independently of Pck1, thereby transmitting cell integrity signaling to activate Pmk1 MAPK.

Interestingly, Rho1 controls Pmk1 basal activity during vegetative growth and cell wall damage, and Rho5 is a functional orthologue of Rho1 (Toda *et al.* 1996; Sengar *et al.* 1997; Arellano *et al.* 1999; Calonge *et al.* 2000; Sanchez-Mir *et al.* 2014). Therefore, although Rho5 deletion only moderately affected the phosphorylation of Pmk1 upon heat stress, it would be intriguing to speculate that Rho5 may play a role in the regulation of the Pmk1 pathway in the stress conditions, because Rho5 expression was heavily induced in the stationary phase (Rincon *et al.* 2006).

It should be noted however, that deletion of Rho4/Rho5 and Pmk1 was additive in terms of their effects on the *vic* phenotype. These results suggested that although Rho4 and Rho5 small G proteins are involved in the Pmk1-mediated cell wall integrity pathway, they may exert their function through yet unidentified target(s) involved in the regulation of Pmk1 MAPK signaling. In support of this view, a significant fraction of Rho4 and Rho5 was observed to localize to internal membranes in addition to the plasma membrane, suggesting the possibility that Rho4 and Rho5, similar to Cdc42, may play an important role in membrane trafficking to the vacuoles thereby regulating cell wall integrity and/or Pmk1 signaling. In agreement with this idea, Rho4p is involved in the regulation of Eng1p and Agn1p secretion during cytokinesis (Santos *et al.* 2005). Alternatively, Rho4 and Rho5, by localizing to the vacuoles may regulate Ca²⁺ homeostasis, because vacuoles are important stores of Ca²⁺, and modulating Ca²⁺

homeostasis is an additional mechanism that effects the *vic* phenotype in fission yeast (Ma *et al.* 2011; Sugiura *et al.* 2012). Recent work by Philips reported that growth factors stimulated rapid and transient activation of Ras on the plasma membrane followed by delayed and sustained activation on the Golgi, showing that Ras proteins on internal membranes can signal, and thus highlighting the importance on the Ras compartmentalization (Philips 2005). In this regard, the prenylating enzymes FTase and GGTase I play important roles in compartmentalization of Ras family signaling.

In higher eucaryotes, the Ras superfamily of small GTPases is frequently active in cancer and thus an attractive target for cancer therapy (Pylayeva-Gupta *et al.* 2011). Although Ras and Rho proteins, both acting as regulators of cell proliferation and cell morphology, have been recognized as contributors to oncogenesis, recent efforts have focused on the development of effective Ras signaling inhibitors (Baines *et al.* 2011). Because Ras and Rho proteins have common post-translational modifications, inhibitors of prenylation are worthy drug targets. Given the high degree of conservation of enzymes involved in prenylation such as GGTase and FTase, Ras and Rho proteins as well as their involvement in human oncogenesis, the screen for *vic* mutants using a fission yeast model may be useful for studying the conserved molecular mechanism of the compartmentalization of the Ras and Rho superfamily.

Experimental procedures

Strains, media, and genetic and molecular biology methods

S. pombe strains used in this study are listed in Table 1. The complete medium YPD (yeast extract-peptone-dextrose) and the minimal medium EMM (Edinburgh minimal medium) have been described previously (Toda *et al.* 1996). Standard genetic and recombinant-DNA methods (Moreno *et al.* 1991) were used except where noted. FK506 was provided by Astellas Pharma, Inc. (Tokyo, Japan). Genomic DNA clones were provided by the National Bio Resource Project, Yeast Genetic Resource Center (Graduate School of Science, Osaka City University).

Isolation of *vic2-1/cwg2-v2* mutant

The *vic2-1/cwg2-v2* mutant was isolated in a screen of cells that had been mutagenized with nitrosoguanidine as described previously (Zhang *et al.* 2000; Ma *et al.* 2006). Briefly, the mutants were spread on YPD plates to give ~1,000 cells/plate and incubated at 27°C for 4 days. The plates were then replica-plated at 27°C onto plates containing 0.5 µg/ml FK506 and 0.2 M MgCl₂. Mutants that grew in the plates were selected and designated as *vic* (viable in the presence of immunosuppressant and chloride ion) mutants. The original mutants isolated were backcrossed three times to wild-type strains HM123 and HM528.

Cloning of the *vic2⁺/cwg2⁺* gene

To clone the *vic2⁺* gene, the temperature sensitivity of *vic2-1* mutants (KP1978) was utilized. The *vic2-1* mutants were grown at 27°C and transformed with an *S. pombe* genomic DNA library constructed in the vector pDB248 (Beach *et al.* 1982). Leu⁺ transformants were replica-plated onto YPD plates at 36°C and the plasmid DNA was recovered from the transformants that showed plasmid-dependent rescue. These plasmids complemented the temperature sensitivity and *vic* phenotype of the *vic2-1* mutant. By DNA sequencing, the suppressing plasmids were identified to contain the

cwg2⁺ gene (SPAC2E1P5.04c). To investigate the relationship between the cloned *cwg2*⁺ gene and *vic2-1* mutant, linkage analysis was performed as follows: the entire *cwg2*⁺ gene was subcloned into the pUC-derived plasmid containing the *S. cerevisiae* *LEU2* gene and integrated by homologous recombination into the genome of the wild-type strain HM123. The integrant was mated with the *vic2-1* mutant. The resulting diploid was sporulated, and tetrads were dissected. In all cases examined, only parental ditype tetrads were found, indicating allelism between the *cwg2*⁺ gene and the *vic2-1* mutation.

Cloning and tagging of the *rho4*⁺, *rho5*⁺, *cdc42*⁺ and *cwg2*⁺ genes

The *rho4*⁺, *rho5*⁺, *cdc42*⁺, and *cwg2*⁺ genes were amplified by PCR with the genomic DNA of wild-type cells as a template. The primers used were summarized in Table 2. The amplified products containing these genes were digested with *Bam*HI or *Bgl*II, and the resulting fragments were subcloned into BlueScriptSK (+) (Stratagene).

For ectopic expression of proteins, we used the thiamine-repressible *nmt1* promoter (Maundrell 1993). Expression was repressed by the addition of 5 μ M thiamine to EMM, and was induced by washing, and then incubating the cells in EMM lacking thiamine. To express GFP-Rho1, Rho2, Rho4, Rho5 or Cdc42, these genes were tagged at their N-terminus with GFP carrying the S65T mutation. Similarly, Rho1, Rho4, Rho5 or Cdc42 were tagged at their N-terminus with GST.

***cdc42L160S* mutant**

Rincón *et al.* have first reported that a *cdc42L160S* mutation allele causes a temperature-sensitive phenotype of the cell growth in *S. pombe* (Rincon *et al.* 2009). By referring to their work, we introduced a corresponding mutation in *cdc42*⁺ gene as follows. First, a 3.7 kb *Sac*I-segmented fragment of *S. pombe* genomic DNA containing *cdc42*⁺ locus (from -1557 to 5256) was cloned in pT7Blue plasmid according to the manufacturer's protocol (Takara Bio Inc.). Next, we introduced a mutation in this

plasmid pT7gcdc42 by replacing nucleotide sequence, gctttgacc, in the third exon of *cdc42⁺* gene (from 1068 to 1076), that encodes Ala¹⁵⁹ Leu¹⁶⁰ Thr¹⁶¹, with gcgctgacc by using the PrimeSTAR[®] mutagenesis basal kit (Takara Bio Inc.). This mutation changes only Leu¹⁶⁰ to Ser¹⁶⁰ in the primary structure of Cdc42. Besides a preparation of the plasmid pT7gcdc42L160S, we mutagenized pT7gcdc42 in the same way to construct another plasmid in which a nucleotide sequence, gtcaatcacacacttc, on 3' UTR of *cdc42⁺* (from 1332 to 1347) was replaced with gtcgaccggggttaac (underline, *HpaI*-digestive site) and inserted a 1.8 kb DNA fragment containing *ura4⁺* selectable marker gene in the *HpaI* site artificially created in the resultant plasmid. After treating the plasmid pT7gcdc42-ura4 with *SacI*, *ura4D18*-auxotrophic mutant strain was transformed with the digested DNA and was spread on an EMM plate. Transformed strain was then transformed again by pT7gcdc42L160S pretreated with *SacI*, and cells were spread on YEA supplemented with 0.5 mg/mL 5-fluoroorotic acid for isolating a clone that lost *ura4⁺* gene by replacing with a *cdc42L160S* DNA fragment. Finally, we confirmed that no unexpected mutation was generated in ORF of *cdc42⁺* gene in the isolated strain.

Microscopy and miscellaneous methods

Light microscopy methods (e.g., fluorescence microscopy) were performed as described previously (Kita *et al.* 2004). Photographs were taken using AxioImager A1 (Carl Zeiss, Germany) equipped with an AxioCam MRm camera (Carl Zeiss, Germany) and AxioVision software (Carl Zeiss, Germany). Images were processed with the ZEN 2012 software (Carl Zeiss, Germany). FM4-64 and Calcofluor labeling, cell extract preparation and immunoblot analysis were performed as previously described (Kita *et al.* 2004).

Acknowledgments

We would like to thank the Yeast Resource Centre (YGRC/NBRP; <http://yeast.lab.nig.ac.jp/nig>) for providing reagents; William Figoni for critical reading of the manuscript, and Astellas pharma Inc. for gifts of FK506. This work was

supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) to R. S. (23390021) and MEXT (Ministry of Education, Culture, Sports, Science and Technology) -Supported Program for the Strategic Research Foundation at Private Universities (2014-2018) (S1411037). We also would like to express our gratitude to members of the Laboratory of Molecular Pharmacogenomics for their support.

References

- Arellano, M., Coll, P.M., Yang, W., Duran, A., Tamanoi, F. & Perez, P. (1998) Characterization of the geranylgeranyl transferase type I from *Schizosaccharomyces pombe*. *Mol. Microbiol.* **29**, 1357-1367.
- Arellano, M., Duran, A. & Perez, P. (1996) Rho1 GTPase activates the (1-3) β -D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. *EMBO J.* **15**, 4584-4591.
- Arellano, M., Duran, A. & Perez, P. (1997) Localisation of the *Schizosaccharomyces pombe* rho1p GTPase and its involvement in the organisation of the actin cytoskeleton. *J Cell Sci.* **110**, 2547-2555.
- Arellano, M., Valdivieso, M.H., Calonge, T.M., Coll, P.M., Duran, A. & Perez, P. (1999) *Schizosaccharomyces pombe* protein kinase C homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell integrity. *J. Cell Sci.* **112**, 3569-3578.
- Baines, A.T., Xu, D. & Der, C.J. (2011) Inhibition of Ras for cancer treatment: the search continues. *Future Med. Chem.* **3**, 1787-1808.
- Beach, D., Piper, M. & Nurse, P. (1982) Construction of a *Schizosaccharomyces pombe* gene bank in a yeast bacterial shuttle vector and its use to isolate genes by complementation. *Mol. Gen. Genet.* **187**, 326-329.
- Broadus, M.R. & Gould, K.L. (2012) Multiple protein kinases influence the redistribution of fission yeast Clp1/Cdc14 phosphatase upon genotoxic stress. *Mol. Biol. Cell* **23**, 4118-4128.
- Calonge, T.M., Nakano, K., Arellano, M., Arai, R., Katayama, S., Toda, T., Mabuchi, I. & Perez, P. (2000) *Schizosaccharomyces pombe* Rho2p GTPase regulates cell wall α -glucan biosynthesis through the protein kinase Pck2p. *Mol. Biol. Cell* **11**, 4393-4401.
- Carver, P.L. (2004) Micafungin. *Ann. Pharmacother.* **38**, 1707-1721.
- Diaz, M., Sanchez, Y., Bennett, T., Sun, C.R., Godoy, C., Tamanoi, F., Duran, A. & Perez, P. (1993) The *Schizosaccharomyces pombe* *cwg2⁺* gene codes for the β subunit

- of a geranylgeranyltransferase type I required for β -glucan synthesis. *EMBO J.* **12**, 5245-5254.
- Estravis, M., Rincon, S.A., Santos, B. & Perez, P. (2011) Cdc42 regulates multiple membrane traffic events in fission yeast. *Traffic* **12**, 1744-1758.
- Herskowitz, I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell* **80**, 187-197.
- Kita, A., Sugiura, R., Shoji, H., He, Y., Deng, L., Lu, Y., Sio, S.O., Takegawa, K., Sakaue, M., Shuntoh, H. & Kuno, T. (2004) Loss of Apm1, the μ 1 subunit of the clathrin-associated adaptor-protein-1 complex, causes distinct phenotypes and synthetic lethality with calcineurin deletion in fission yeast. *Mol. Biol. Cell* **15**, 2920-2931.
- Lane, K.T. & Beese, L.S. (2006) Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J. Lipid Res.* **47**, 681-699.
- Levin, D.E. & Errede, B. (1995) The proliferation of MAP kinase signaling pathways in yeast. *Curr. Opin. Cell Biol.* **7**, 197-202.
- Ma, Y., Kuno, T., Kita, A., Asayama, Y. & Sugiura, R. (2006) Rho2 is a target of the farnesyltransferase Cpp1 and acts upstream of Pmk1 mitogen-activated protein kinase signaling in fission yeast. *Mol. Biol. Cell* **17**, 5028-5037.
- Ma, Y., Sugiura, R., Koike, A., Ebina, H., Sio, S.O. & Kuno, T. (2011) Transient receptor potential (TRP) and Cch1-Yam8 channels play key roles in the regulation of cytoplasmic Ca^{2+} in fission yeast. *PLOS ONE* **6**, e22421.
- Madrid, M., Soto, T., Khong, H.K., Franco, A., Vicente, J., Perez, P., Gacto, M. & Cansado, J. (2006) Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **281**, 2033-2043.
- Marshall, C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet. Dev.* **4**, 82-89.
- Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for

- fission yeast. *Gene* **123**, 127-130.
- Moreno, S., Klar, A. & Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795-823.
- Munshi, A. & Ramesh, R. (2013) Mitogen-activated protein kinases and their role in radiation response. *Genes cancer* **4**, 401-408.
- Nakano, K., Arai, R. & Mabuchi, I. (1997) The small GTP-binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe*. *Genes Cells.* **2**, 679-694.
- Nakano, K., Arai, R. & Mabuchi, I. (2005) Small GTPase Rho5 is a functional homologue of Rho1, which controls cell shape and septation in fission yeast. *FEBS letters* **579**, 5181-5186.
- Nakano, K., Mutoh, T., Arai, R. & Mabuchi, I. (2003) The small GTPase Rho4 is involved in controlling cell morphology and septation in fission yeast. *Genes Cells.* **8**, 357-370.
- Nishida, E. & Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128-131.
- Philips, M.R. (2005) Compartmentalized signalling of Ras. *Biochem. Soc. Trans.* **33**, 657-661.
- Pylayeva-Gupta, Y., Grabocka, E. & Bar-Sagi, D. (2011) RAS oncogenes: weaving a tumorigenic web. *Nat. Rev. Cancer* **11**, 761-774.
- Ribas, J.C., Diaz, M., Duran, A. & Perez, P. (1991) Isolation and characterization of *Schizosaccharomyces pombe* mutants defective in cell wall (1-3) β -D-glucan. *J. Bacteriol.* **173**, 3456-3462.
- Rincon, S.A., Santos, B. & Perez, P. (2006) Fission yeast Rho5p GTPase is a functional paralogue of Rho1p that plays a role in survival of spores and stationary-phase cells. *Eukaryot. Cell* **5**, 435-446.
- Rincon, S.A., Ye, Y., Villar-Tajadura, M.A., Santos, B., Martin, S.G. & Perez, P. (2009) Pobl participates in the Cdc42 regulation of fission yeast actin cytoskeleton. *Mol. Biol. Cell.* **20**, 4390-4399.

- Rincon, S.A., Estravis, M. & Perez, P. (2014) Cdc42 regulates polarized growth and cell integrity in fission yeast. *Biochem. Soc. Trans.* **42**, 201-205.
- Sanchez-Mir, L., Soto, T., Franco, A., Madrid, M., Viana, R.A., Vicente, J., Gacto, M., Perez, P. & Cansado, J. (2014) Rho1 GTPase and PKC ortholog Pck1 are upstream activators of the cell integrity MAPK pathway in fission yeast. *PLOS ONE* **9**, e88020.
- Santos, B., Gutierrez, J., Calonge, T.M. & Perez, P. (2003) Novel Rho GTPase involved in cytokinesis and cell wall integrity in the fission yeast *Schizosaccharomyces pombe*. *Eukaryot. cell* **2**, 521-533.
- Santos, B., Martin-Cuadrado, A.B., Vazquez de Aldana, C.R., del Rey, F. & Perez, P. (2005) Rho4 GTPase is involved in secretion of glucanases during fission yeast cytokinesis. *Eukaryot. Cell* **4**, 1639-1645.
- Satoh, R., Tanaka, A., Kita, A., Morita, T., Matsumura, Y., Umeda, N., Takada, M., Hayashi, S., Tani, T., Shinmyozu, K. & Sugiura, R. (2012) Role of the RNA-binding protein Nrd1 in stress granule formation and its implication in the stress response in fission yeast. *PLOS ONE* **7**, e29683.
- Sengar, A.S., Markley, N.A., Marini, N.J. & Young, D. (1997) Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **17**, 3508-3519.
- Sugiura, R., Toda, T., Shuntoh, H., Yanagida, M. & Kuno, T. (1998) *pmp1⁺*, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast. *EMBO J.* **17**, 140-148.
- Sugiura, R., Kita, A., Tsutsui, N., Muraoka, O., Hagihara, K., Umeda, N., Kunoh, T., Takada, H. & Hirose, D. (2012) Acremomannolipin A, the potential calcium signal modulator with a characteristic glycolipid structure from the filamentous fungus *Acremonium strictum*. *Bioorg. Med. Chem. Lett.* **22**, 6735-6739.
- Takada, H., Nishimura, M., Asayama, Y., Mannse, Y., Ishiwata, S., Kita, A., Doi, A., Nishida, A., Kai, N., Moriuchi, S., Tohda, H., Giga-Hama, Y., Kuno, T. & Sugiura, R. (2007) Atf1 is a target of the mitogen-activated protein kinase Pmk1 and regulates cell integrity in fission yeast. *Mol. Biol. Cell* **18**, 4794-4802.
- Takai, Y., Sasaki, T. & Matozaki, T. (2001) Small GTP-binding proteins. *Physiol Rev.* **81**,

153-208.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R. & Kuno, T. (1996) The fission yeast *pmk1⁺* gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. *Mol. Cell. Biol.* **16**, 6752-6764.

Zaitsevskaya-Carter, T. & Cooper, J.A. (1997) Spm1, a stress-activated MAP kinase that regulates morphogenesis in *S.pombe*. *EMBO J.* **16**, 1318-1331.

Zhang, Y., Sugiura, R., Lu, Y., Asami, M., Maeda, T., Itoh, T., Takenawa, T., Shuntoh, H. & Kuno, T. (2000) Phosphatidylinositol 4-phosphate 5-kinase Its3 and calcineurin Ppb1 coordinately regulate cytokinesis in fission yeast. *J. Biol. Chem.* **275**, 35600-35606.

Figure Legends

Figure 1. A mutation in the *vic2*⁺/*cwg2*⁺ gene causes *vic* and temperature-sensitive phenotypes. (A) The *vic* and temperature-sensitive phenotypes of *vic2-1/cwg2-v2* mutant cells. Cells transformed with the multicopy vector pDB248 or the vector containing the *cwg2*⁺ gene were streaked onto the plates as indicated, then incubated for 4 days at 27°C or 3 days at 36°C, respectively. (B) Partial alignment of protein sequences of *S. pombe* Cwg2 with related proteins from human (PGGT1B), and mouse (Pgg1b). Sequence alignment was performed using the Clustal W program. Asterisks indicate identical amino acids and colons indicate similar amino acids. Arrow indicates the mutation site in the arginine 254 of Cwg2, which when mutated to lysine resulted in *vic* and temperature-sensitive phenotypes in Cwg2. (C) Genetic interaction between Pmk1 and Cwg2. The cells as indicated were dropped onto the plate as indicated, then incubated for 4 days at 27°C. (D) The *cwg2-v2* cells are hypersensitive to β-glucanase treatment. Cells as indicated were grown in YPD at 27 °C and treated with 0.3 mg/mL Zymolyase-100T for various times, and the OD₆₆₀ of the cell suspension was monitored. (E) Fluorescent micrographs of Calcofluor-stained *cwg2-v2*, $\Delta pmk1$, *cwg2-v2* $\Delta pmk1$ and wt cells incubated at 27°C as indicated. Bar, 10 μm. Right panel; Septation index and percentage of multi-septated cells from three independent experiments in *cwg2-v2*, $\Delta pmk1$, *cwg2-v2* $\Delta pmk1$ and wt cells incubated at 27°C. At least 150 cells were observed at one time in each experiment. Bars, SD.

Figure 2. The *cwg2-v2* mutation affects the intracellular localization of multiple Rho GTPases. (A) The C-terminal sequence of Rho1, Rho2, Rho3, Rho4, Rho5 and Cdc42 in fission yeast. Rho1, Rho4, Rho5 and Cdc42 contain CAAX motif modified by GGTase. (B) Intracellular localization of various small GTPases in wild-type (wt) and *cwg2-v2* mutant cells. The GFP-fused Rho1, Rho4, Rho5, Cdc42 or Rho2 was transformed into wild-type and *cwg2-v2* mutant cells. The transformants were grown

to early log phase in EMM containing 5 μ M thiamine, and were examined under the fluorescence microscope. Bar, 10 μ m. (C) Vacuolar localization of various Rho proteins in wt and *cwg2-v2* mutant cells. Cells as indicated in Fig. 2B were loaded with FM4-64 for 60 min and suspended with water for 60 min and then examined by fluorescent microscopy. Bar, 10 μ m.

Figure 3. Inactivation of Rho4 and Rho5 induced *vic* phenotype. Inactivation of Rho4 and Rho5 induced *vic* phenotype. Knockout of the *rho4*⁺, *rho5*⁺, and the component of the Pmk1 MAPK pathway exhibited *vic* phenotype and micafungin sensitivity. The cells as shown were streaked onto the plates as indicated, then incubated for 4 days at 27°C.

Figure 4. Rho4 and Rho5 function upstream of the Pmk1 MAPK pathway. (A) The Cwg2-Rho4/Rho5 module is implicated in the activation of Pmk1 *in vivo*. Wild-type (wt), *cwg2-v2*, Δ *rho4* or Δ *rho5* cells expressing Pmk1-GST from the endogenous promoter were grown in EMM and treated with heat shock (42°C) for 0, 10, or 20 min. Immunoblotting using anti-phospho Pmk1 (anti-phos. Pmk1) and anti-GST antibodies showed that Cwg2, Rho4, as well as Rho5 are required for the sufficient activation of Pmk1 upon heat shock. Upper panel: The data shown are representative of three independent experiments. Lower panel: Quantification of Pmk1 phosphorylation. The data were averaged from three independent experiments. Bars, SD.

(B) Rho4 and Rho5 stimulate the phosphorylation of Pmk1 *in vivo*. Wild-type cells expressing Pmk1-GST from the endogenous promoter, transformed with either control vector (+ vector), pREP1-Rho4 (+ Rho4), or pREP1-Rho5 (+ Rho5), were grown in EMM. Cells were incubated for 24 hr in EMM without thiamine to induce the overexpression of Rho4 and Rho5. Immunoblotting using anti-phospho Pmk1 (anti-phos. Pmk1) and anti-GST (lower panel) antibodies showed that overproduction of

Rho4, as well as Rho5, but not that of control vector increased the levels of phosphorylation of Pmk1. Upper panel: The data shown are representative of three independent experiments. Lower panel: Quantification of Pmk1 phosphorylation. The data were averaged from three independent experiments. Bars, SD.

Figure 5. Genetic interaction between Rho4/Rho5 and Pmk1. (A) $\Delta rho4\Delta pmk1$ and $\Delta rho5\Delta pmk1$ double mutants showed synergism in the tolerance of $MgCl_2$ in the presence of FK506, but not in the sensitivity to micafungin. Wild-type, $\Delta pmk1$, $\Delta rho4$, $\Delta rho5$, $\Delta rho4\Delta pmk1$ or $\Delta rho5\Delta pmk1$ cells were dropped onto the plates as indicated, then incubated for 4 days at 27°C. (B) The cells as indicated in (A) were dropped onto the plates containing a lower concentration of micafungin, and incubated for 4 days at 27°C. (C) The sensitivities of $\Delta pmk1$, $\Delta rho4$, $\Delta rho5$, $\Delta rho4\Delta pmk1$ or $\Delta rho5\Delta pmk1$ cells to the cell wall lytic enzyme β -glucanase. Cells as indicated were treated with β -glucanase as in Fig. 1D. (D) Cytokinesis abnormality in the knockout of Rho4/Rho5-Pmk1 signaling module. Calcofluor staining of the cells as indicated in (A) and (B). Bar, 10 μm . Right panel: Septation index and percentage of multi-septated cells from three independent experiments in $\Delta rho4$, $\Delta rho5$, $\Delta rho4\Delta pmk1$, $\Delta rho5\Delta pmk1$ and wt cells. At least 150 cells were observed at one time in each experiment. Bars, SD.

Figure 6. Physical interaction between Rho4/Rho5 and Pcks. (A-B) Pck1 and 2 associate with Rho4 and Rho5. GST pull-downs performed with GST-Pck1 or GST-Pck2; cells expressing GFP alone, GFP-Rho1, GFP-Rho4, or GFP-Rho5 were collected, and the lysates were incubated with purified GST-Pck1 or GST-Pck2. Proteins bound to glutathione beads were analyzed by SDS-PAGE and immunoblotted using anti-GFP or anti-GST antibodies.

Figure S1. Comparison of the impact of each Rho protein on Pmk1 signaling. (A) $\Delta rho2$ cells displayed the severest micafungin sensitivity and the strongest *vic*

phenotype. The cells as shown were streaked onto the plates as indicated, then incubated for 4 days at 27°C. (B) Wild-type cells expressing Pmk1-GST from the endogenous promoter, transformed with either control vector (+ vector), pREP1-Rho1 (+ Rho1), pREP1-Rho2 (+ Rho2), pREP1-Rho3 (+ Rho3), pREP1-Rho4 (+ Rho4), or pREP1-Rho5 (+ Rho5), were grown in EMM. Cells were incubated for 20 hr in EMM without thiamine to induce the overexpression of each Rho protein. Immunoblotting using anti-phospho Pmk1 (anti-phos. Pmk1) and anti-GST (lower panel) antibodies showed that overproduction of Rho2, but not that of other Rho protein or the control vector increased the levels of phosphorylation of Pmk1. The data shown are representative of three independent experiments.

Figure S2. Overexpression of *pck1*⁺, but not *pck2*⁺, *rho4*⁺, or *rho5*⁺, partially suppressed the micafungin sensitivity of Δ *pmk1* cells. The Δ *pmk1* or Δ *mkh1* cells were transformed with the control vector, *pck1*⁺ *pck2*⁺, *rho4*⁺, or *rho5*⁺ gene. The transformants were dropped onto the plates as indicated and then incubated for 3 d at 27°C.

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Reference
HM123	<i>h</i> ⁻ <i>leu1-32</i>	Our stock
HM528	<i>h</i> ⁺ <i>his2</i>	Our stock
KP928	<i>h</i> ⁺ <i>his2 leu1 ura4-D18</i>	Our stock
KP1978	<i>h</i> ⁻ <i>leu1-32 cwg2-v2</i>	This study

KP208	<i>h⁻ leu1-32 ura4-D18 pmk1::ura4⁺</i>	Our stock
KP2178	<i>h⁻ leu1-32 pmk1::KanMX6</i>	Our stock
KP2780	<i>h⁻ leu1-32 ura4-D18 rho2::ura4⁺</i>	Our stock
KP1375	<i>h⁻ leu1-32 ura4-D18 rho3::ura4⁺</i>	Our stock
SP439	<i>h⁻ leu1-32 ura4-D18 ade6-M216 rho4::ura4⁺</i>	Nakano <i>et al.</i> 2003
SP504	<i>h⁻ leu1-32 ura4-D18 rho4::ura4⁺</i>	This study
SP2402	<i>h⁺ leu1-32 ura4-D18 ade6-M216 rho5::ura4⁺</i>	Nakano <i>et al.</i> 2005
SP2428	<i>h⁻ leu1-32 ura4-D18 rho5::ura4⁺</i>	This study
SP2440	<i>h⁺ leu1-32 ura4-D18 ade6-M216 cdc42L160S</i>	This study
SP2455	<i>h⁺ leu1-32 cdc42L160S</i>	This study
SP2423	<i>h⁻ leu1-32 ura4-D18 rho4::ura4⁺ pmk1::KanMX6</i>	This study
SP2429	<i>h⁻ leu1-32 ura4-D18 rho5::ura4⁺ pmk1::KanMX6</i>	This study
SP2437	<i>h⁻ leu1-32 cwg2-v2 pmk1::KanMX6</i>	This study
SP2231	<i>h⁻ leu1-32 pmk1⁺ GST::KanMX6</i>	This study
SP2441	<i>h⁻ leu1-32 rho4::ura4⁺ pmk1⁺ GST::KanMX6</i>	This study
SP2449	<i>h⁻ leu1-32 rho5::ura4⁺ pmk1⁺ GST::KanMX6</i>	This study

Table 2. *Schizosaccharomyces pombe* primers used in this study

Gene	Primer
Rho4 sense	5'- GAAGATCTCATGTCTGCTTTCAAAAAGAGTGGATCC -3'
Rho4 antisense	5'- GAAGATCTCTTATAAAAATCACGCAAGACTTTTTAG -3'
Rho5 sense	5'- CGGGATCCATGACTACTGAGCTCCGTCGTAAATTGG -3'
Rho5 antisense	5'- CGGGATCCTCAGAGTAAGATACAGTGCTTCTTC -3'
Cdc42 sense	5'- CGCGGATCCCATATGCCCACCATTAAGTGTGTCGTAG -3'
Cdc42 antisense	5'- CGCGGATCCTTACAGTACCAAACACTTTGAC -3'
Cwg2 sense	5'- GCGTTACAGCCCCTTCGGCGCCTAGTAAAGTGTTGC -3'
Cwg2 antisense	5'- GCAACACTTTACTAGGCGCCGAAGGGGCTGTAACGC -3'

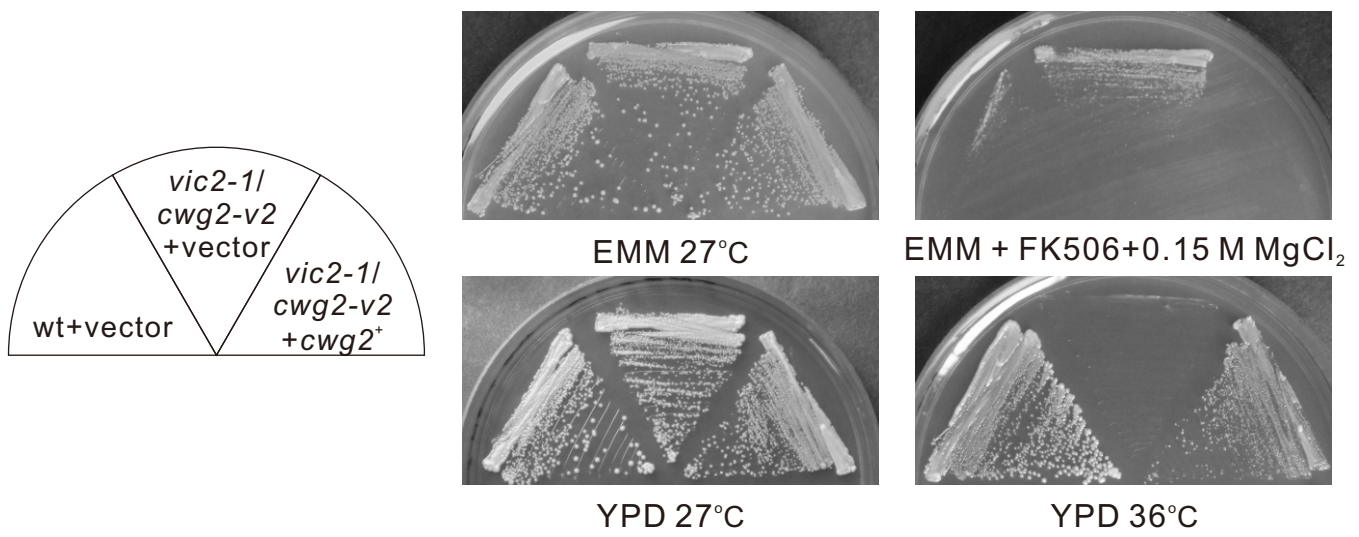
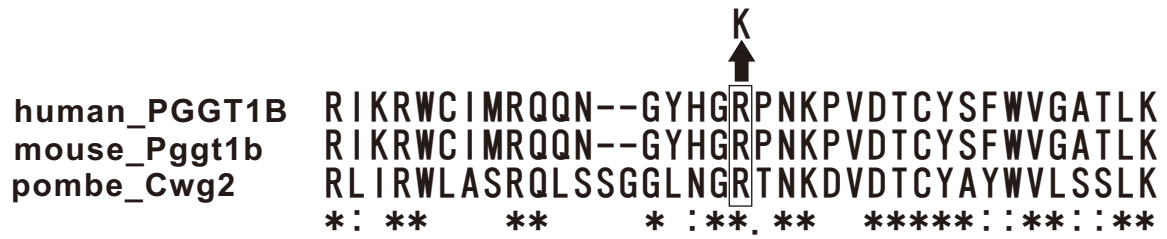
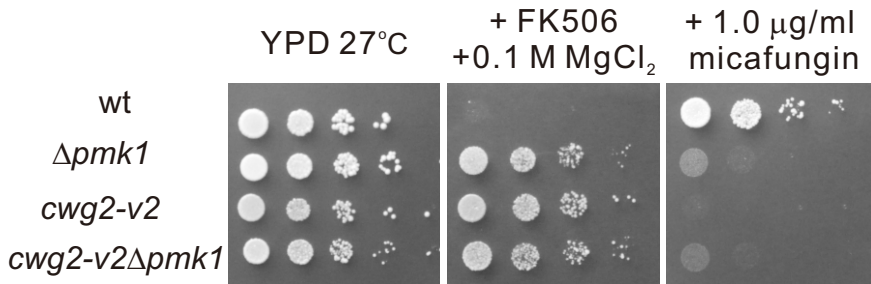
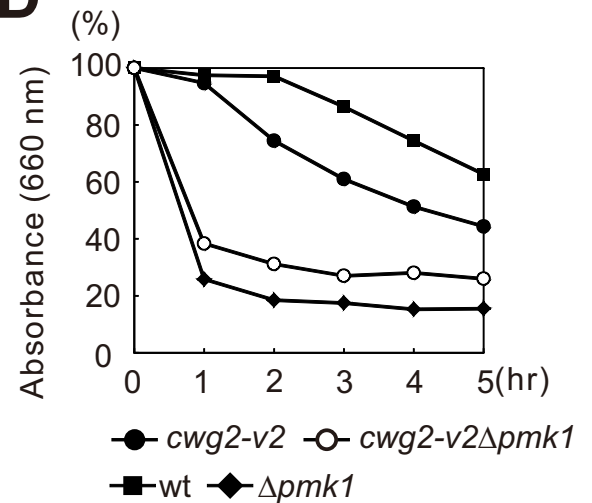
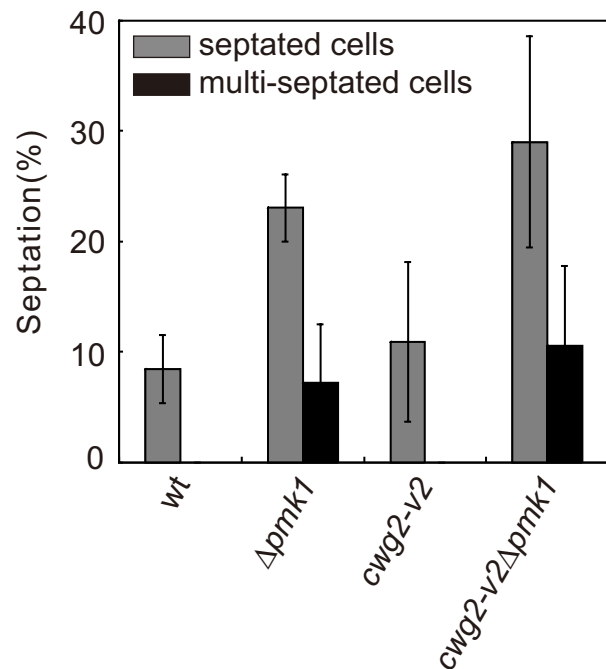
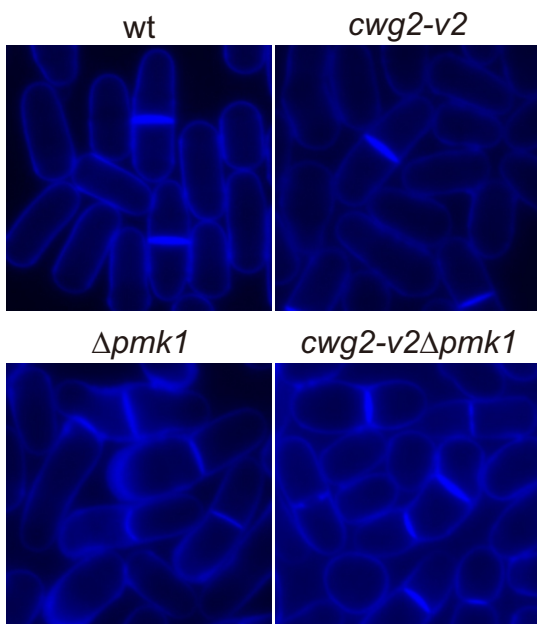
A**B****C****D****E**

Figure 1

A

Rho1	CILL	motif modified by GGTase1
Rho2	CIIS	motif modified by FTase
Rho3	CIIA	motif modified by FTase
Rho4	CVIL	motif modified by GGTase1
Rho5	CILL	motif modified by GGTase1
Cdc42	CLVL	motif modified by GGTase1

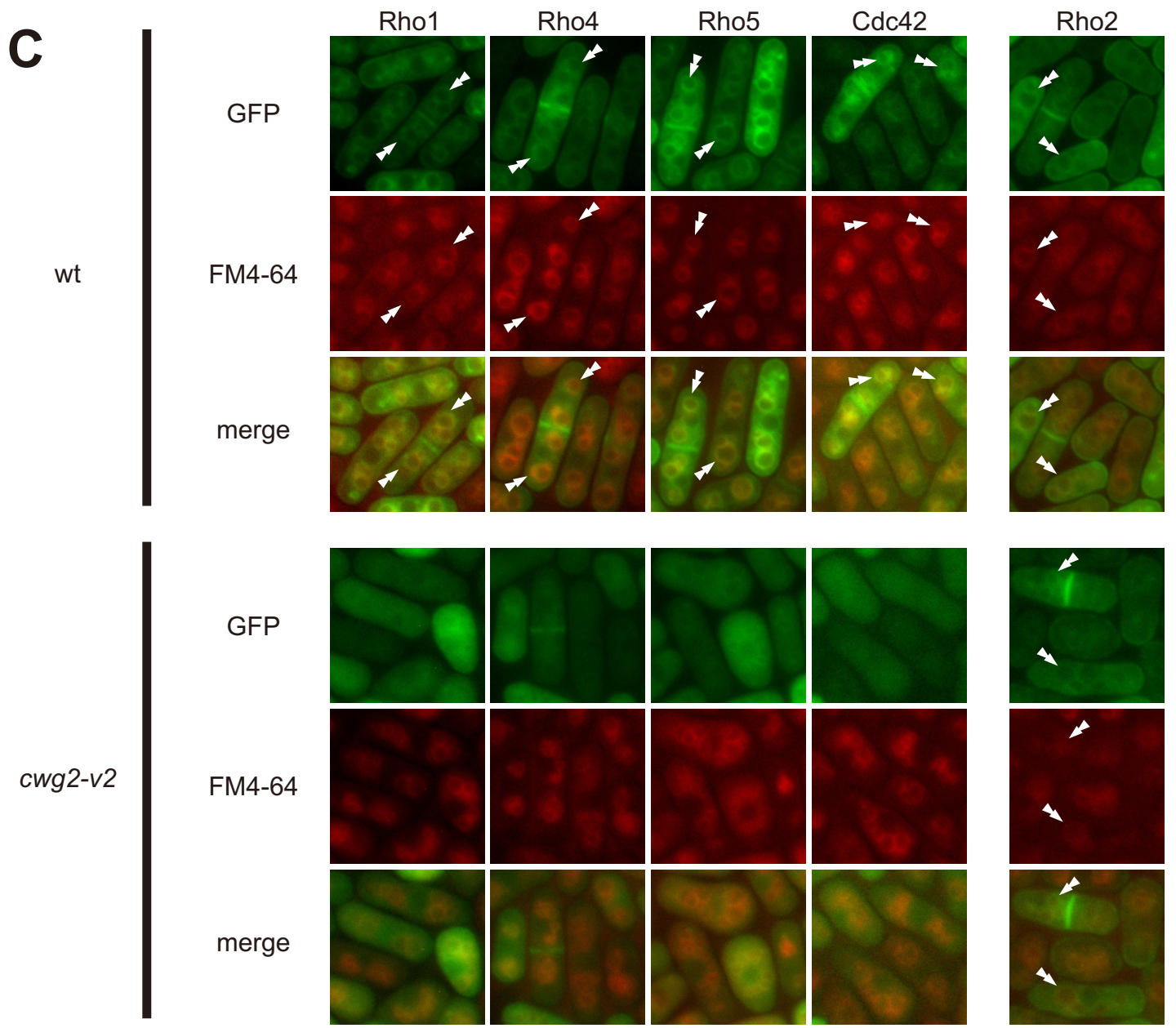
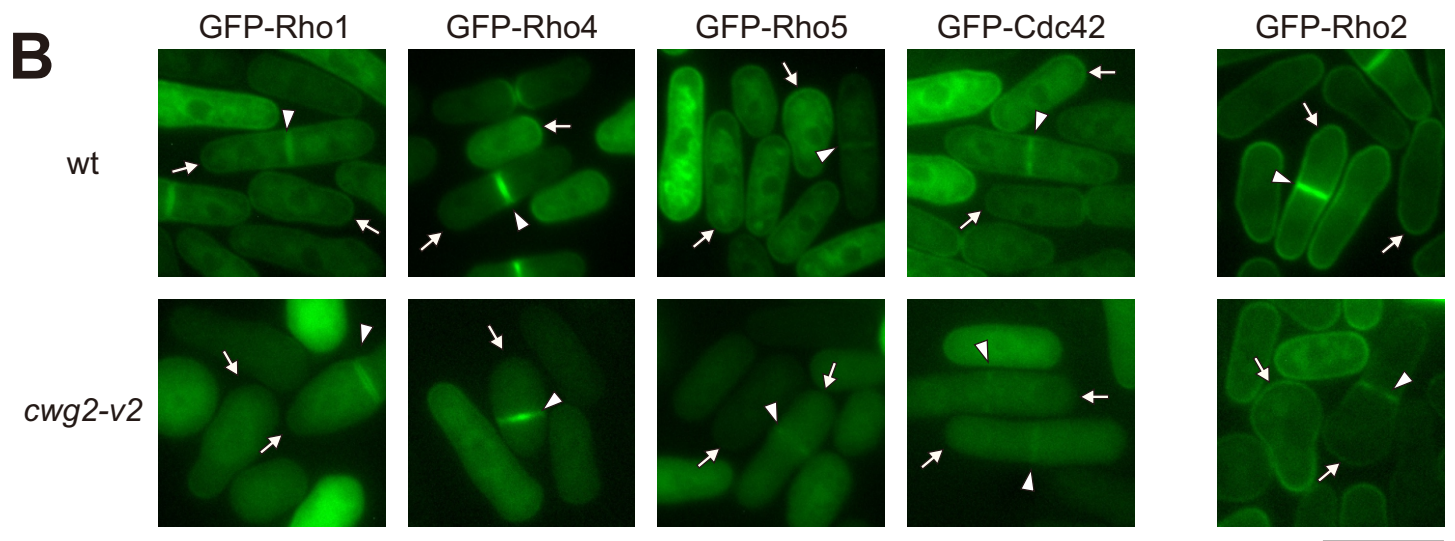
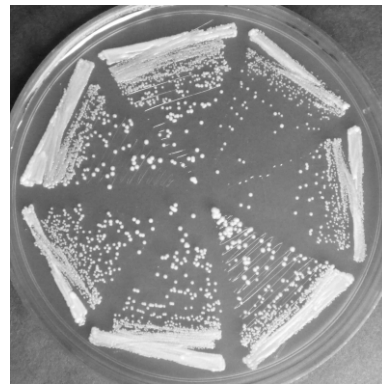
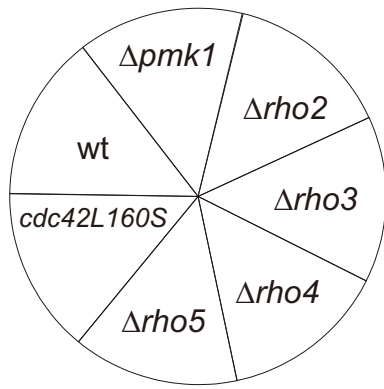
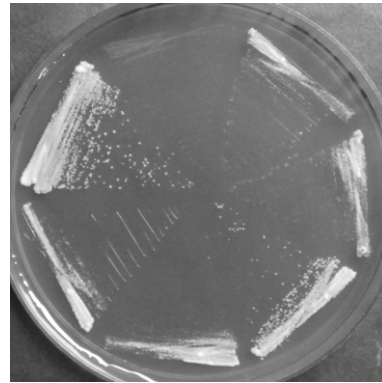
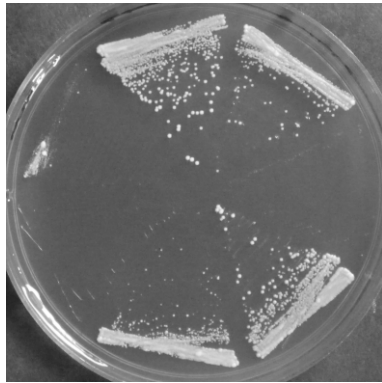


Figure 2



YPD 27°C



YPD + FK506+0.1 M MgCl₂ YPD + 1.0 μg/ml micafungin

Figure 3

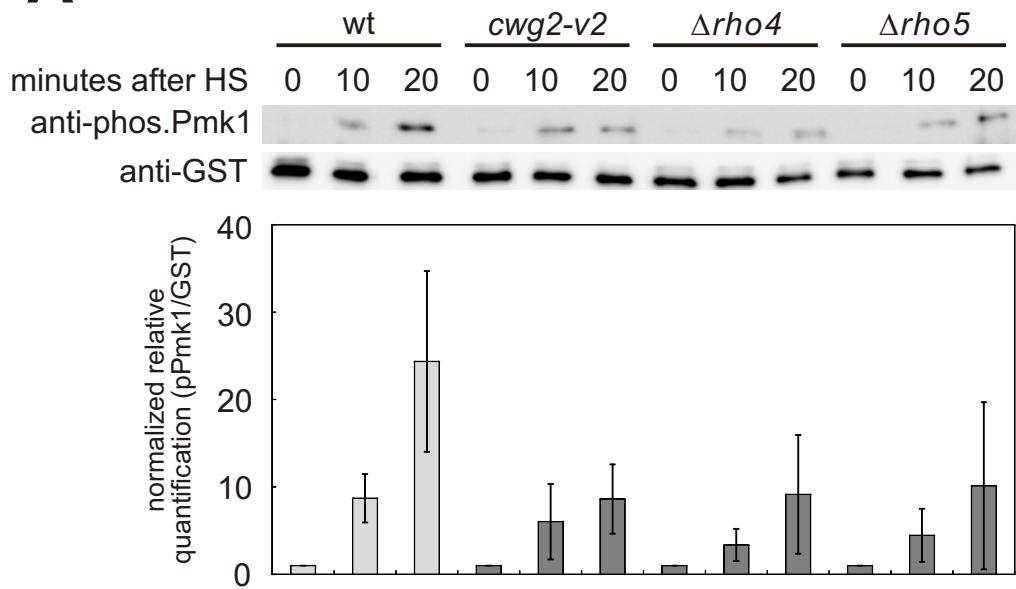
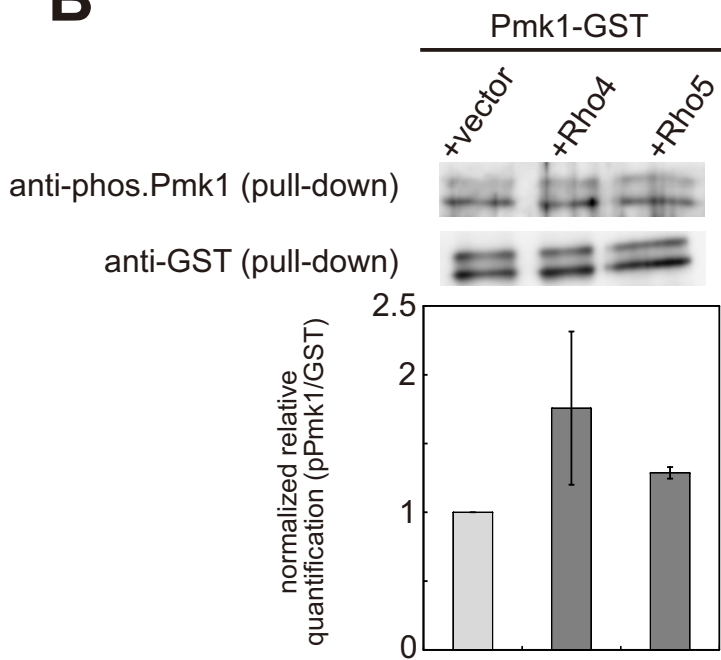
A**B**

Figure 4

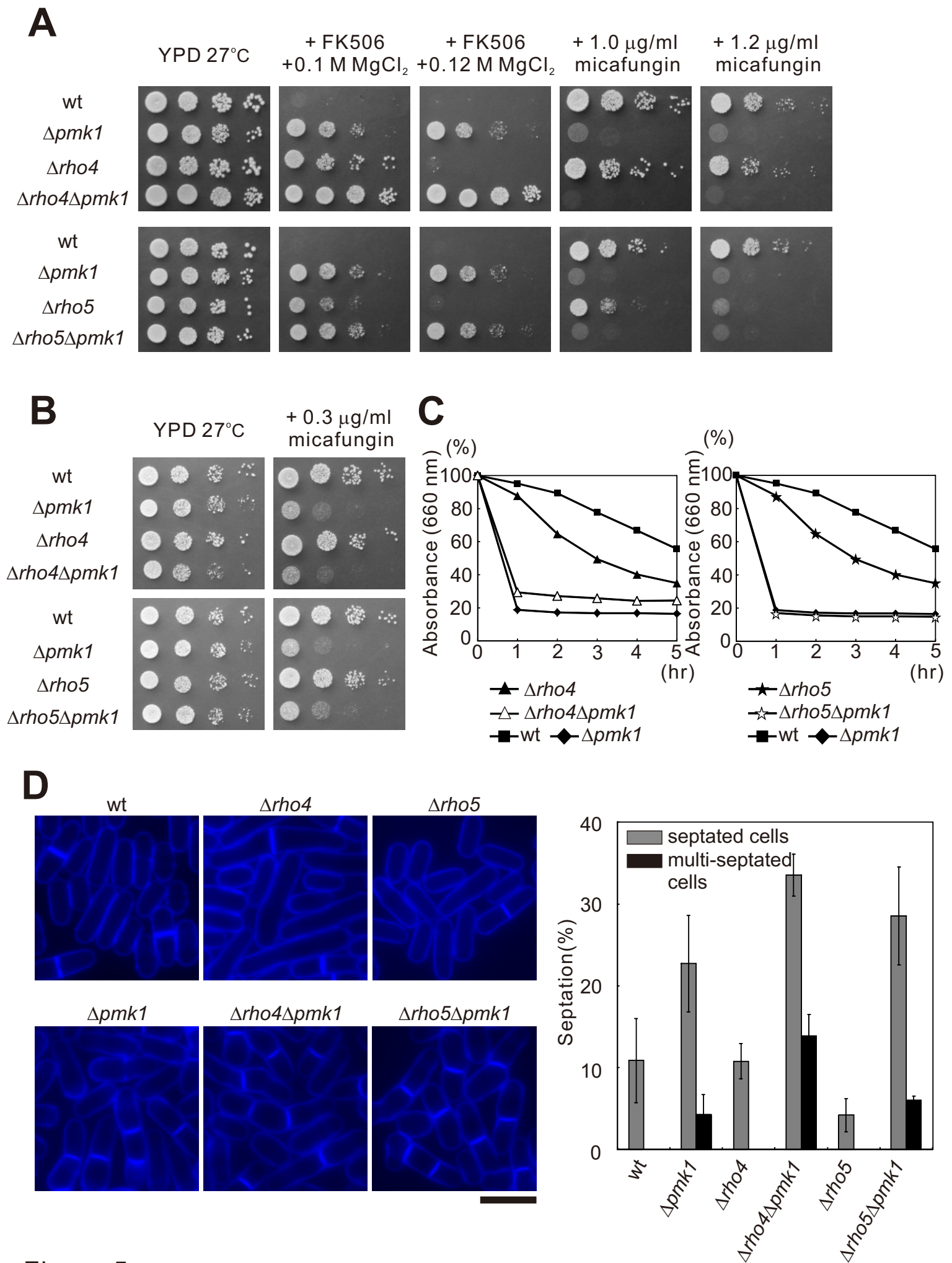


Figure 5

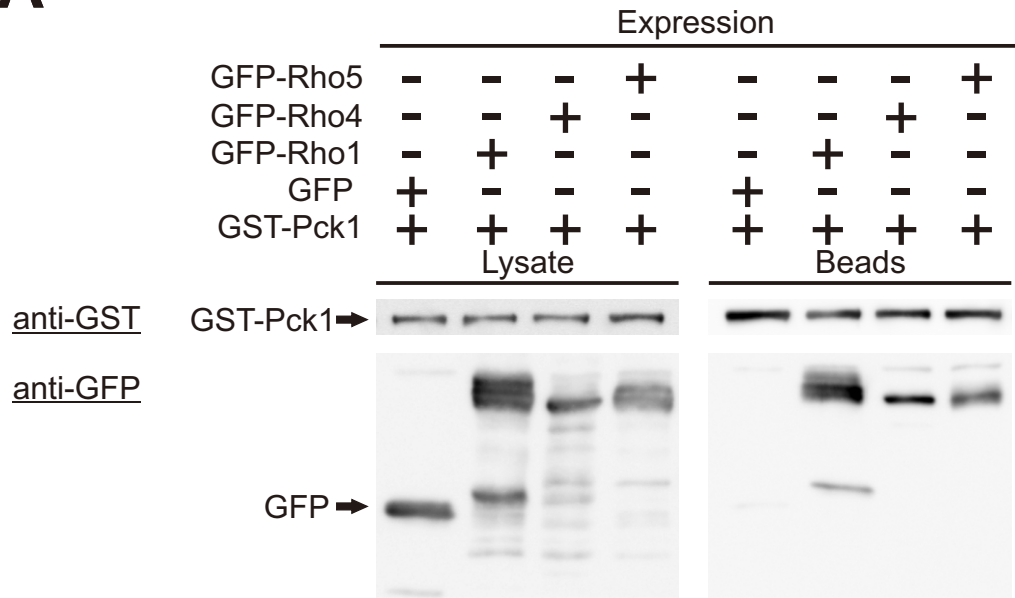
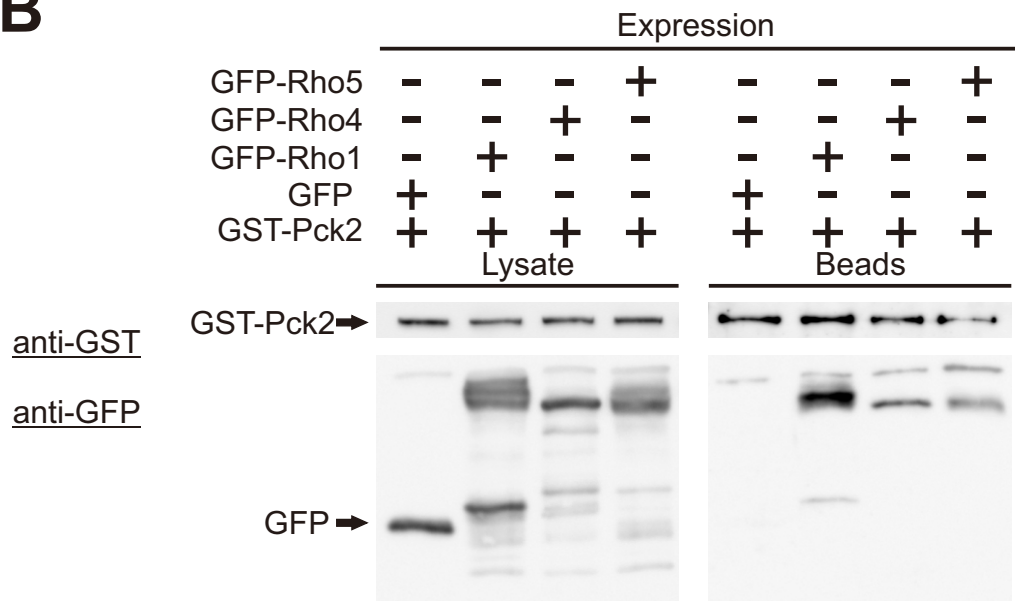
A**B**

Figure 6

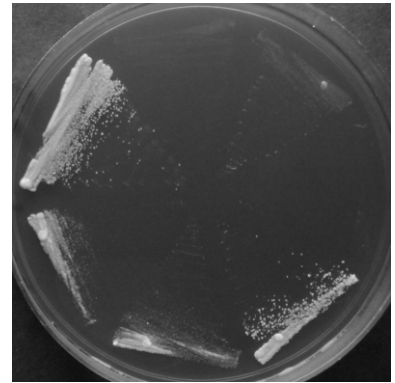
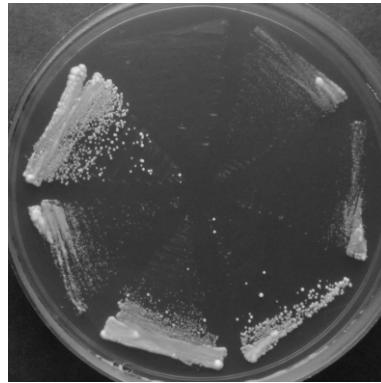
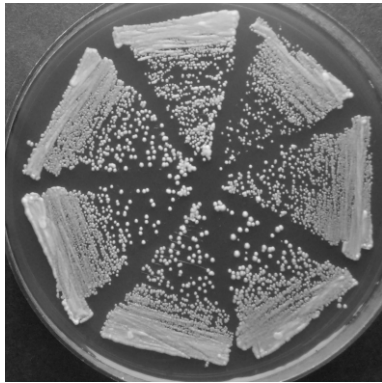
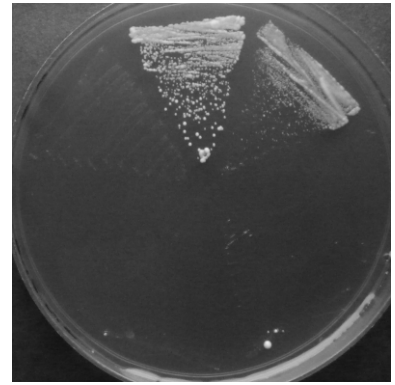
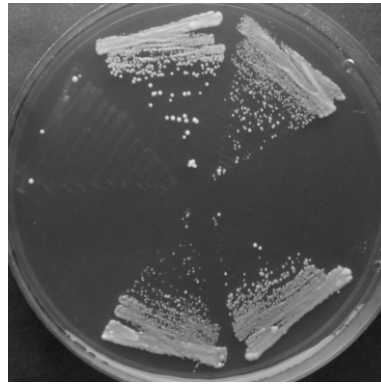
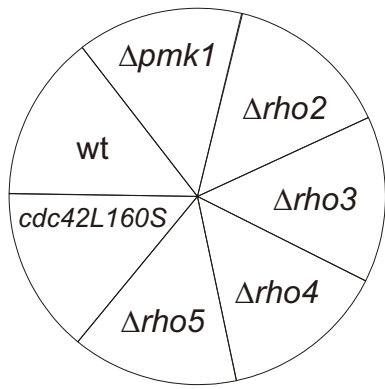
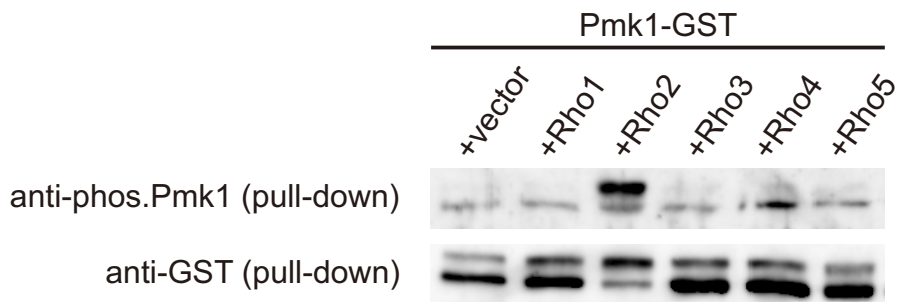
A**B**

Figure S1

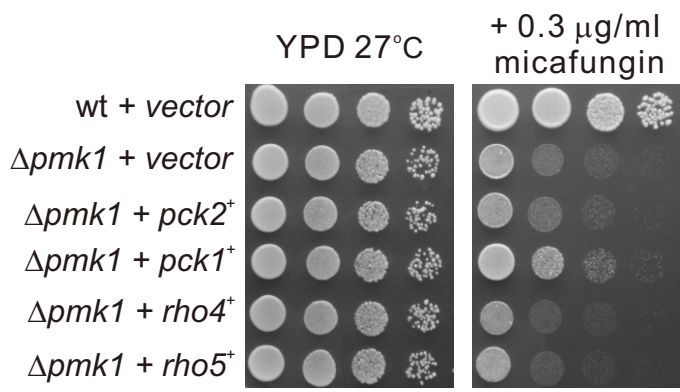


Figure S2