

# Rho5p downregulates the yeast cell integrity pathway

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## Summary

The Rho family of proteins and their effectors are key regulators involved in many eukaryotic cell functions. In *Saccharomyces cerevisiae* the family consists of six members, Rho1p to Rho5p and Cdc42p. With the exception of Rho5p, these enzymes have been assigned different biological functions, including the regulation of polar growth, morphogenesis, actin cytoskeleton, budding and secretion. Here we show that a *rho5* deletion results in an increased activity of the protein kinase C (Pkc1p)-dependent signal transduction pathway. Accordingly, the deletion shows an increased resistance to drugs such as caffeine, Calcofluor white and Congo red, which indicates

activation of the pathway. In contrast, overexpression of an activated *RHO5Q91H* mutant renders cells more sensitive to these drugs. We conclude that Rho5p acts as an off-switch for the MAP-kinase cascade, which differentiates between MAP-kinase-dependent and -independent functions of Pkc1p. Kinetics of actin depolarisation and repolarisation after heat treatment of *rho5* deletions as well as strains overexpressing the activated *RHO5Q91H* allele provide further evidence for such a function.

Key words: *Saccharomyces cerevisiae*, MAP kinase, *RHO5*, Signal transduction, GTPase

## Introduction

Cellular integrity in the yeast *Saccharomyces cerevisiae* depends on the proper composition of the cell wall and is controlled by the Pkc1p-mediated signal transduction through a MAP kinase cascade (reviewed by Heinisch et al., 1999). This pathway is generally activated by events that lead to a change of the cell's size or shape. Early work in this area was focused primarily on studies of the regulation of cell wall biosynthesis. However it soon became evident that the pathway is also important for processes such as cell fusion (Philips and Herskowitz, 1997), polarized growth (Andrews and Stark, 2000), regulation of actin cytoskeleton polarisation (Delley and Hall, 1999), control of the morphogenesis checkpoint during cell cycle (Harrison et al., 2001) and several other events that affect cell membrane or cell wall composition. Signals activating the pathway are detected by sensors located at the cell surface, such as Slg1p/Wsc1p (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998) and Mid2p (Rajavel et al., 1999; Ketela et al., 1999). Information is then transduced via the GDP/GTP exchange factor Rom2p to the small GTPase Rho1p (Ozaki et al., 1996). The latter, like all small GTPases, is considered active in its GTP-bound and inactive in its GDP-bound state (Drgonova et al., 1996; Nonaka et al., 1995). Sac7p and Lrg1p act as GAPs (GTPase-activating protein) for Rho1p and thus function as negative regulators (Watanabe et al., 2001; Lorberg et al., 2001). Further GAP functions have been assigned to Bag7p and Bem2p (Peterson et al., 1994; Schmidt et al., 1997) but seem to be less crucial for Rho1p function (Lorberg et al., 2001).

Similar to many other small GTPases, Rho1p has a set of different target proteins. In its GTP-bound state it binds to and thereby activates the  $\beta$ -1,3-glucan synthase complex (Drgonova et al., 1996). It is also involved in regulation of the actin cytoskeleton by interacting with Bni1p (Fujiwara et al.,

1998). In addition, interaction with Skn7p, a regulator of oxidative stress response (Krems et al., 1996; Alberts et al., 1998), has been reported. With respect to signals ensuring cellular integrity, the main effector of Rho1p is the yeast protein kinase C homologue Pkc1p (Nonaka et al., 1995). This kinase then activates a MAP-kinase cascade consisting of the MAPKKK Bck1p (Lee and Levin, 1992), the MAPKKs Mkk1p and/or Mkk2p (Irie et al., 1993) and the MAPK Slt2p (Torres et al., 1991), also referred to as Mpk1p (Lee et al., 1993b). Rlm1p (Watanabe et al., 1995) and the SBF complex (consisting of Swi4p and Swi6p) (Madden et al., 1997) have been reported as targets of the MAP kinase Slt2p. Rlm1p then regulates transcription of a specific set of genes (Jung and Levin, 1999).

Apart from signalling through the MAPK cascade Pkc1p activity has been related to the regulation of a variety of cascade-independent functions, including oligosaccharyl-transferase activity (Park and Lennarz, 2000), ribosomal gene transcription (Li et al., 2000), nuclear perturbation caused by high osmolarity (Nanduri and Tartakoff, 2001), microtubule function (Hosotani et al., 2001) and regulation of phospholipid synthesis (Sreenivas et al., 2001).

In addition, Pkc1p is involved in controlling the dynamics of actin cytoskeleton organisation. In budding yeast cells the cortical actin patches are polarized correlating with directed growth of the cells (Adams and Pringle, 1984; Lew and Reed, 1995; Amberg, 1998). With the change to isotropic growth at mitosis the patches redistribute over the bud and mother cell surface and then reorient after cytokinesis to the mother-bud junction. The asymmetric distribution of actin patches can be disturbed by different stresses such as hypertonic media or heat shock (Chowdhury et al., 1992; Lillie and Brown, 1994). The repolarisation of actin during adaptation to growth at higher temperatures has been shown to be an event dependent on

Pkc1p but not on Slf2p activity (Delley and Hall, 1999). This is especially interesting because this repolarisation is antagonistic to the depolarisation of the actin cytoskeleton that occurs upon the shift to high temperatures. The latter is also mediated by Pkc1p but in contrast to the repolarisation it requires an active MAP-kinase cascade (Delley and Hall, 1999).

In addition to Rho1p, five other homologues encoding members of the Rho family were identified in the *S. cerevisiae* genome (Garcia-Ranea and Valencia, 1998) namely *RHO2*, *RHO3*, *RHO4*, *RHO5* and *CDC42*. Only *CDC42* and *RHO1* are essential genes. Cdc42p participates in the establishment of cell polarity and bud site assembly (Johnson and Pringle, 1990) as well as in a late step in exocytosis (Adamo et al., 2001). Rho3p and Rho4p are also involved in exocytosis and affect actin cytoskeleton assembly (Adamo et al., 1999). Very little information is available on the function of Rho5p. Until now no phenotype could be assigned either to its gene deletion or to its overexpression (Roumanie et al., 2001). Three proteins were found to interact with Rho5p: Med11p and Ynl099p were identified in two high-throughput interaction studies (Schwikowski et al., 2000; Ito et al., 2001); an additional interactor, Rgd2p was isolated in a systematic screen for GAP functions (Roumanie et al., 2001). Although the latter finding proved that Rho5p in vivo acts as a small GTPase, no reasonable hint as to its intracellular role has been provided to date. Because it is known from higher eukaryotes that different

Rho-type GTPases can function in the same pathway (Anastasiadis and Reynolds, 2001), we speculated that, like Rho1p, Rho5p may be involved in the Pkc1p/cell wall integrity pathway of *S. cerevisiae*.

The data we present in this work indicate that the signal generated by Rho5p has a negative effect on the output of the MAP kinase branch of the Pkc1p-mediated signal transduction pathway.

## Materials and Methods

### Genetic manipulations and sequencing

Standard molecular genetic procedures were followed in the preparation and handling of DNA (Sambrook et al., 1989). Transformation of yeast cells was performed by the lithium acetate method (Gietz et al., 1995), and plasmids from yeast cells were isolated as described (Robzyk and Kassir, 1992). Sequencing was performed by the dideoxy-chain termination method using the Sequenase version 2.0 from Amersham Pharmacia in conjunction with [<sup>35</sup>S]dATP using standard primers. Alternatively, custom sequences were obtained from SeqLab (Göttingen, Germany).

### Plasmids, primers and in vitro mutagenesis

Plasmids and primers used in this study are listed in Tables 1 and 2, respectively. In vitro mutagenesis (Boles and Miosga, 1995) was carried out using primer RHO5Q91Hneu, M13 reverse primer and pUC19RHO5hin as a template to generate the megaprimer. The

**Table 1. Plasmids**

pUC19	Yanisch-Perron et al., 1985.
pUG6	Used as a source for the G418 resistance marker located between direct repeats of the <i>loxP</i> site (Güldener et al., 1996).
pFA6- <i>HIS3MX6</i>	Used as a source for the heterologous <i>Sphis5+</i> marker (Longtine et al., 1998).
pHPS100	Vector carrying both the information for a <i>lexA-Rlm1p_N</i> fusion protein and the corresponding <i>lacZ</i> reporter gene (Kirchrath et al., 2000).
pJH447	The <i>GALI/10</i> promoter was amplified by PCR using the oligonucleotide pair GAL1atg and GAL10atg (Table 2) from genomic DNA prepared from strain VW1A (Lorberg et al., 1999) and subcloned as an <i>EcoRI/BamHI</i> fragment into the polylinker site of YEp352 (Hill et al., 1986).
pUC19RHO5her	<i>RHO5</i> was amplified by PCR from genomic <i>S. cerevisiae</i> DNA using RHO5-3neu and RHO5-5neu as primers and digested with <i>BglIII</i> and <i>BclI</i> . The resulting 2.0 kb fragment was ligated into pUC19 digested with <i>BamHI</i> . Transcription of <i>RHO5</i> proceeds in opposite direction to that of <i>lacZ</i> .
pUC19RHO5hin	The same PCR generated fragment as in pUC19RHO5her was inserted in the opposite orientation into pUC19.
pSH4	<i>RHO5</i> was amplified by PCR from pUC19RHO5hin using RHO5Bam and RHO5-3'Sph as primers and then digested with <i>BamHI</i> and <i>SphI</i> . The resulting 1.2 kb fragment was ligated into pJH447 digested with <i>BamHI</i> and <i>SphI</i> .
pHPS116	The 2.1 kb product of the PCR mutagenesis generating the activated <i>RHO5</i> allele (see 'In vitro mutagenesis' in Materials and Methods) was digested with <i>SphI</i> and ligated into pUC19 digested with the same enzyme. Transcription of the modified <i>RHO5</i> gene proceeds in the same direction as that of the <i>lacZ</i> gene.
pSH8	Vector carrying the activated <i>RHO5</i> allele under the control of the <i>GALI/10</i> promoter. Obtained from an in vivo recombination of pSH4 (6.8 kb <i>PstI/Asp718I</i> fragment) and pHPS116 (2.1 kb <i>SphI</i> fragment).

**Table 2. Oligonucleotides used in this study**

delRHO5-5	5'-CGC CGA AAT ATT TAT TAA ATA CAT ATA GTA TAC TAA TAA GTC TGC TTC GTA CGC TGC AGG TCG AC-3'
delRHO5-3	5'-CGC CGA AAT ATT TAT TAA ATA CAT ATA GTA TAC TAA TAA GTC TGC TTC GTA CGC TGC AGG TCG AC-3'
RHO5-5neu	5'-CCA CAT CGG TAA GTG ATG ACC-3'
RHO5-3neu	5'-CCA TTA CTA TGG TTC AAC CTG G-3'
RHO5Q91Hneu	5'-CAT AAC GGT CTT AAA CGA TCG TAA TCT TCG TGT CCT GCG GTG TCC CAT AAA TTG-3'
RHO5Bam	5'-GGA TGG <u>ATC</u> CAT GAG GTC TAT TAA ATG TGT G-3'
RHO5-3eSph	5'-GGA TGC <u>ATG</u> CTT GGA AAA GAA AGT CGC GG-3'
delBEM2-5	5'-GGA TTG TAT ACA TTT ACC ACG AAA ATT GTT TAT TGC TTG AAA TAATCC ATA GGC CAC TAG TGG ATC TG-3'
delBEM2-3	5'-GGA CAA TTC ATC TCT TTT CCT GCG GTT ATG AGC GAA ACC AAC GTT GCT TCG TAC GCT GCA GGT CGA C-3'
GAL1atg	5'-GGC <u>GAA</u> <u>TTC</u> TAT AGT TTT TTC TCC TTG ACG-3'
GAL10atg	5'-CTC <u>GGA</u> <u>TCC</u> TTA TAT TGA ATT TTC-3'
M13 reverse	5'-CAG GAA ACA GCT ATG ACC ATG-3'

Restriction sites used for subcloning are underlined.

product was purified and used in a second PCR again with M13 reverse primer but together with pUC19RHO5her as a template. This resulted in an exchange of a T at position 263 of the *RHO5* open reading frame for a C but did not alter the amino acid sequence. Instead it removes a *Pst*I site to distinguish between the wild-type and the mutated allele. Additionally the G at position 273 was exchanged for a C to alter the coding sequence at amino acid position 91 from glutamine to histidine.

#### Video microscopy

The video microscopy setup consisted of an Axioplan 2 microscope (Carl Zeiss AG, Feldbach, Switzerland), equipped with a 75W/XBO epifluorescence illumination source, a motorized stage and a Plan-Neofluar 100×/1.3 Oil PH3 objective. HiQ filter sets were used (Chroma Technology, Brattleboro, VT). Because the spectrum of a XBO lamp shows a considerable emission in the infrared range we doubled the normal heat absorption and reflection filters in the microscope in order to protect the specimen. Fluorescence excitation was controlled by a shutter controller in combination with a MAC2000 shutter system (Ludl controller MAC 2000, Ludl Electronics, Hawthorne, CA). We used a TE/CCD-1000PB back-illuminated cooled CCD camera mounted on the primary port in combination with the ST-133 controller (Princeton Instruments, Trenton, NJ). Microscope, camera and fluorescence shutter were controlled by the Metamorph v. 3.51 software (Universal Imaging, West Chester, PA). Five Z-axis planes spaced by 0.5 μm were taken for each fluorescence image and one plane was taken as a phase-contrast image from every sample. The resulting pictures were deblurred using the 2D deconvolution function of the Autodeblur 6.0 (Autoquant Imaging, Watervliet, NY). After deconvolution the pictures were retransferred into Metamorph and the fluorescence images were combined using the maximum option. Phase-contrast and fluorescence pictures were then combined using the overlay command assigning a red look-up table (LUT) for the phase-contrast and a green look-up table (LUT) for the fluorescence images. Final size, contrast and brightness of the pictures was adjusted using Adobe PhotoShop 6.0.

#### Strains and media

*E. coli* strain DH5αF' (Gibco BRL, Gaithersburg, MD) was used throughout this study. *S. cerevisiae* strains are listed in Table 3. The

*rho5* deletion was constructed using one step gene targeting with pUG6 (Güldener et al., 1996) and primers delRHO5-5 and delRHO5-3. The *bem2* deletion was built similarly but using primers delBEM2-3, delBEM2-5 and pFA6-*HIS3mx6* (Longtine et al., 1998) as a template. Standard media and growth conditions were employed (Sherman et al., 1986). Rich media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with 2% glucose (YEPD) or galactose. Yeast transformants were selected on minimal medium (0.67% yeast nitrogen base, 2% glucose or galactose) supplemented with amino acids as described (Zimmermann, 1975), and omitting uracil, tryptophane, leucine or histidine when selecting for the respective markers. To test for Pkc1p pathway-related phenotypes Calcofluor white, caffeine and Congo red were added to the media at the concentrations indicated. For serial-dilution patch tests, cells were grown overnight in rich medium with 1 M sorbitol, diluted to OD<sub>600</sub> 0.2 and grown to OD<sub>600</sub> 0.8. Then dilutions as indicated in the figures were made and spotted onto the plates.

#### β-galactosidase assay

For liquid β-galactosidase assays cells were grown overnight. Cells were diluted to an OD<sub>600</sub> of about 0.2 and grown to an OD<sub>600</sub> of between 0.6 and 1.0. Cells were washed once with Z-buffer (16 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 6 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 0.1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O) and the pellet was resuspended in 150 μl of Z-buffer containing 270 μl β-mercaptoethanol per 100 ml. 50 μl of chloroform and 20 μl of 0.1% SDS were added. The samples were vortexed vigorously for 30 seconds. 700 μl of ONPG solution (Z-buffer with β-mercaptoethanol and 1 mg/ml o-Nitrophenyl β-D-galactopyranosid) were added and the samples were incubated at 30°C. The reactions were stopped by addition of 500 μl 1M Na<sub>2</sub>CO<sub>3</sub>. Samples were collected by centrifugation at maximum speed in a microcentrifuge for 10 minutes and the optical density of the supernatant at 420 nm was determined. The activity was calculated by the formula: (OD<sub>420</sub> × 1000) / (OD<sub>600</sub> × reaction time × reaction volume).

#### Actin staining

For actin staining, yeast cells were grown in glucose containing minimal medium overnight. Cells were inoculated into 50 ml of fresh minimal medium containing the carbon source indicated and grown for about 4 hours to an optical density between 0.3 and 0.6 at 30°C.

**Table 3. Yeast strains**

DHD5	<i>MATα</i> <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 MAL/MAL SUC/SUC GAL/GAL</i>	Arvanitidis and Heinisch, 1994
HD261-1A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP MAL GAL SUC</i>	This study
HSH1-1A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP slt2::LEU2 MAL GAL SUC</i>	This study
HD252	<i>MATα</i> <i>ura3-52/ura3-52 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1::loxP/trp1::loxP rho5::loxP-KanMX-loxP/RHO5 MAL/MAL GAL/GAL SUC/SUC</i>	This study
HSH1-1C	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP MAL GAL SUC</i>	This study
HSH1-4A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP MAL GAL SUC</i>	This study
HSH1-1A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP slt2::LEU2 MAL GAL SUC</i>	This study
HSH1-3A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP slt2::LEU2 MAL GAL SUC</i>	This study
HSH1-2B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP MAL GAL SUC</i>	This study
HSH1-7B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP slt2::LEU2 MAL GAL SUC</i>	This study
HSH1-5A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP MAL GAL SUC</i>	This study
HSH1-2A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP slt2::LEU2 MAL GAL SUC</i>	This study
MALY5-3B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP slt2::LEU2 MAL GAL SUC</i>	Lorberg et al., 2001
HSH2-2B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP bem2::Sphis5+ MAL GAL SUC</i>	This study
HSH2-1B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP MAL GAL SUC</i>	This study
HSH2-5A	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP MAL GAL SUC</i>	This study
HSH2-8B	<i>MATα</i> <i>ura3-52 his3-11,15 leu2-3,112 trp1::loxP rho5::loxP-KanMX-loxP bem2::Sphis5+ MAL GAL SUC</i>	This study
PA109-1C	<i>MATα</i> <i>ura3 his3 leu2 trp1 rme1 GAL bck1::HIS3MX6</i>	Delley and Hall, 1999
HPY1-2A	<i>MATα</i> <i>ura3 his3 leu2 trp1 GAL bck1::HIS3MX6</i>	This study
HPY1-2B	<i>MATα</i> <i>ura3 his3 leu2 trp1 GAL</i>	This study
HPY1-2C	<i>MATα</i> <i>ura3 his3 leu2 trp1 GAL bck1::HIS3MX6 rho5::loxP-KanMX-loxP</i>	This study
HPY1-2D	<i>MATα</i> <i>ura3 his3 leu2 trp1 GAL rho5::loxP-KanMX-loxP</i>	This study

To observe actin dynamics under heat shock the cultures were shifted to 37°C. At the times indicated, 10 ml of culture were taken and cells were fixed by addition of 4% formaldehyde to the medium with subsequent incubation for 1 hour at 30°C with gentle shaking. After fixation the yeasts were washed twice with 0.1 M sodium phosphate buffer (pH 7.0) and resuspended in 50 µl of the same buffer. To this, 2.5 µl of a 3.3 µM Alexa phalloidine solution in methanol (Molecular Probes, Eugene, OR) and 2.5 µl of 1% Triton X-100 stock were added and the mixture was incubated for 30 minutes on ice. Finally cells were washed three times with 0.1 M sodium phosphate buffer. 5 µl of the cell suspension on a polylysine-treated microscopy slide under a coverslip were investigated under the microscope.

#### Slt2p phosphorylation assay

The determination of Slt2p phosphorylation was carried out as described previously (Martin et al., 2000) with some modifications (Lorberg et al., 2001).

## Results

### A *rho5* deletion shows increased activity of the cellular integrity pathway

The activity of the Slt2p MAP-kinase pathway can be determined indirectly by the use of a *RLM1/lexA-lacZ* reporter system placed on a centromeric vector (Kirchrath et al., 2000). In this system the activity of the cell integrity pathway is reflected by the β-galactosidase activity. The results of such measurements for a *rho5* deletion mutant (HD261-1A) are presented in Table 4. Clearly, β-galactosidase activities are increased in  $\Delta rho5$  when compared with wild-type cells. Thus, already at 25°C (i.e. under non-activating conditions) the mutant shows β-galactosidase levels about threefold higher than the wild-type. This phenomenon is further enhanced at higher temperature, with about a sixfold increase in activity observed at 37°C.

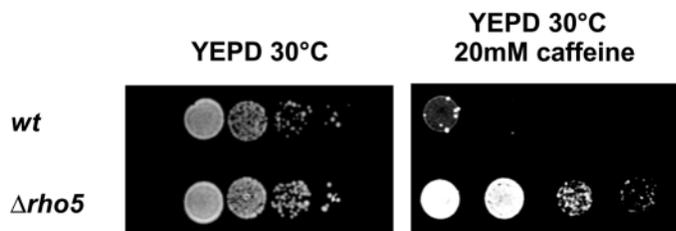
### A *rho5* deletion strain exhibits increased caffeine resistance

The observed increase in activity of the Slt2p MAP-kinase pathway in the *rho5* deletion mutant could be explained in two different ways: (1) the deletion could result in an impaired cellular integrity that in turn is monitored by the sensors of the pathway signalling for an increased activity; or (2) the deletion could affect a function that normally downregulates the pathway. In order to differentiate between these possibilities we determined the sensitivity of the deletion mutants to different drugs or increased growth temperatures. As observed previously (Roumanie et al., 2001), our *rho5* deletion strain

**Table 4. Indirect determination of Slt2p MAP-kinase activity in a *rho5* deletion background**

Growth temperature	Wildtype	$\Delta rho5$
25°C	0.7±0.3	2.3±1.2
37°C	8.4±5.3	50.5±22.7

The activity of the Slt2p MAP-kinase cascade was determined using the plasmid-based reporter system described previously (Kirchrath et al., 2000); for details, see Materials and Methods. The mean and standard deviation values are shown for four different transformants.



**Fig. 1.** A *rho5* deletion shows increased resistance against caffeine. Serial dilutions of strains HSH1-2B (*wt*) and HD261-1A ( $\Delta rho5$ ) were spotted onto the media indicated and incubated at 30°C for 2 days.

grows normally at 37°C (data not shown). Therefore we also tested for sensitivity against caffeine (Fig. 1). Interestingly we observed an increased resistance of the mutant to the drug when compared with the isogenic wild-type strain. Thus, in contrast to the wild-type control, the *rho5* deletion proved to be resistant to doses of up to 20 mM caffeine. Together with the results on pathway activity reported above, this phenotype indicates a role for *RHO5* in the downregulation of the Slt2p MAP-kinase pathway.

### Phenotypes of an activated *rho5* mutant

Mutants defective in the components of the Slt2p-MAPK pathway usually display sensitivity against drugs such as caffeine, Calcofluor white and Congo red (Yoshida et al., 1992; Kirchrath et al., 2000). To provide further evidence for Rho5p being a negative regulator of this pathway we tested the effect of overexpression of an activated allele of *RHO5* for sensitivity against these drugs. For this purpose, we took advantage of the high conservation of essential residues between the members of the Ras superfamily of small GTPases. Mutations in specific residues have been reported to result in hyperactive enzymes. Thus the exchange of glutamine at position 61 in loop 4 of human p21 Ras leads to reduced intrinsic GTP hydrolysis with a constitutively active protein (Krengel et al., 1990). To construct a similar allele for *RHO5* we selected the corresponding residue (glutamine 91) of *RHO5* and exchanged it for a histidine by in vitro mutagenesis (for details, see Materials and Methods). The resulting allele was cloned under the control of the *GALI/10* promoter on a multicopy plasmid to investigate the effect of overexpression (Fig. 2). Cells grown on galactose (i.e. under inducing conditions) did not grow at 37°C and were sensitive against 0.15 mg/ml Calcofluor white, 7 mM caffeine and 0.3 mg/ml Congo red. These overexpression phenotypes of a constitutively active allele are again compatible with Rho5p acting as a negative regulator of the Slt2p MAP-kinase pathway.

### Partial complementation of kinase module defects by a *rho5* deletion

We proceeded with an epistatic analysis of a *rho5* deletion by combining it with other pathway mutants. A double deletion of *bck1* (the MAPKK-kinase) and *rho5* did not suppress the temperature sensitivity of the *bck1* mutant, but rather exacerbated this phenotype (Fig. 3A). By contrast, we observed a suppression of the caffeine sensitivity of the *bck1* deletion.

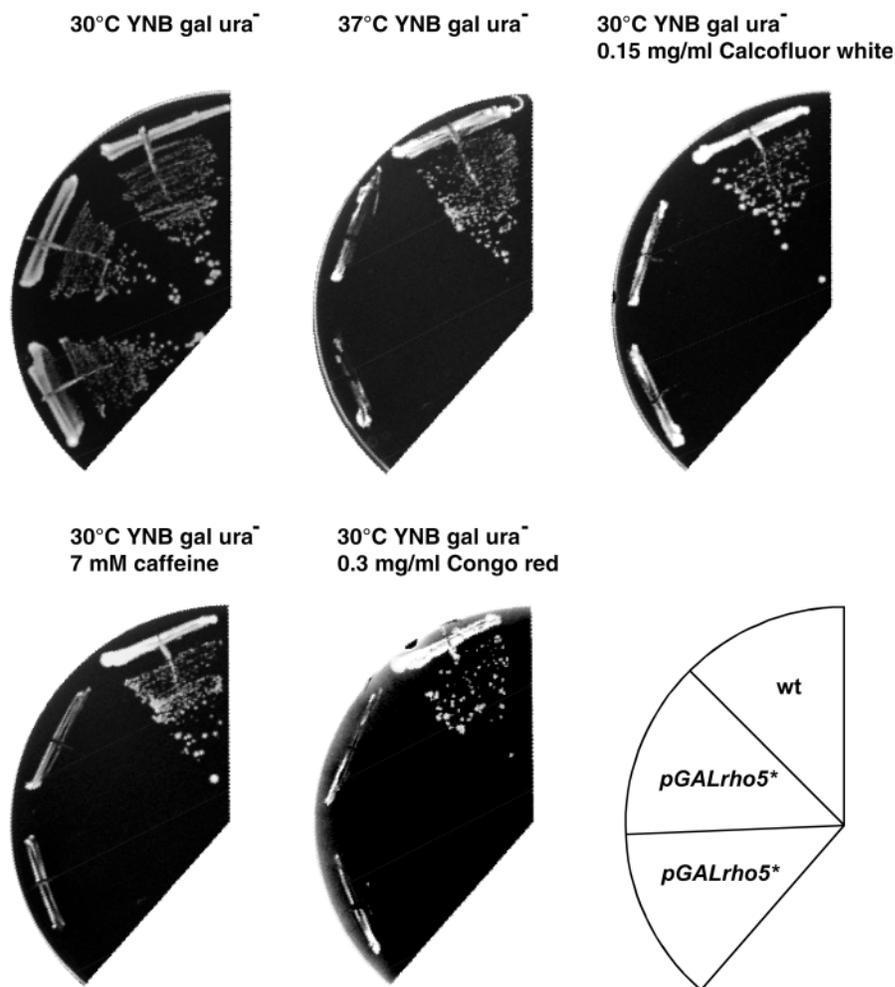
These seemingly contradictory phenotypes could be explained by Rho5p acting downstream of Bck1p but as well by Rho5p being part of a parallel pathway or by a 'crosstalk' with upstream components (for details, see Discussion). Therefore we also tested the effect of a  $\Delta rho5$  deletion in a *slt2* deletion background (encoding the MAP-kinase of the pathway). The double deletion grows at 30°C without osmotic stabilization in contrast to the single *slt2* deletion strain (Fig. 3B). However, no suppression was observed when tested for growth at 37°C. Likewise, the caffeine sensitivity also remained unaltered (data not shown). The suppression of the growth defect of the *slt2* deletion at 30°C suggests that Rho5p functions downstream of the MAP-kinase cascade. Failure to suppress under the other growth conditions indicates that Rho5p may serve additional functions at 37°C, and in the presence of caffeine, that are exerted independently from the MAP-kinase cascade.

#### Slt2p phosphorylation in a *rho5* deletion

The MAPK Slt2p is activated by a dual phosphorylation catalyzed by the MAPKK pair Mkk1p/Mkk2p (Martin et al., 2000). To investigate this kind of pathway regulation by the Rho5p signal, we determined Slt2p phosphorylation in a western blot analysis. Based on the reporter assay described above, where we observed maximal activation at 37°C, we chose this growth condition for pathway activation. As shown in Fig. 4 the amount of phosphorylated Slt2p is not significantly increased in the *rho5* mutant when compared with the wild-type. This substantiates the findings of the suppression experiments described above and places the action of Rho5p downstream of Slt2p but upstream of Rlm1p (i.e. explaining the increased activity in the reporter system).

#### Regulation of Rho5p

Bem2p has been proposed to serve a GTPase-activating function for Rho1p as the upstream activator of protein kinase C (Peterson et al., 1994). Although its deletion showed the expected increase in Slt2p phosphorylation (Martin et al., 2000) the phenotypes of *bem2* deletions are contrary to those expected for an activated Slt2p pathway. Mutants are sensitive to several drugs known to destabilize the cell wall and show a depolarized actin cytoskeleton (Cid et al., 1998). These defects can be suppressed neither by overproduction nor deletion of *SLT2* (Cid et al., 1998). Having shown that Rho5p acts downstream of Slt2p, we also tested whether a *rho5* deletion affects any of the phenotypes of a *bem2* deletion. As shown in

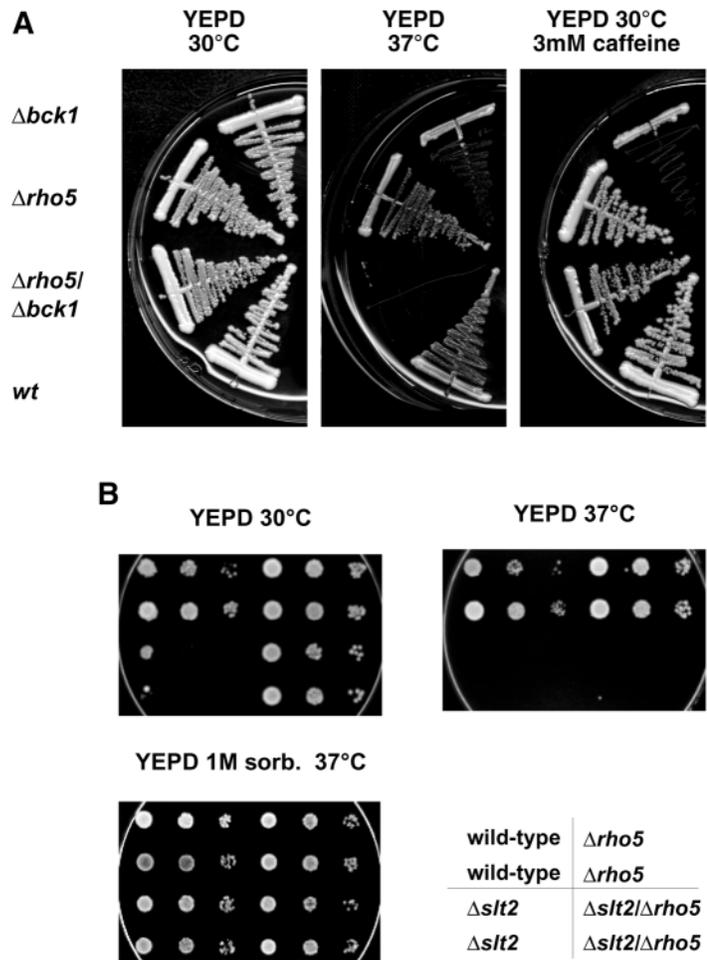


**Fig. 2.** Phenotypes of *RHO5Q91H* overexpression. Cells were grown on glucose-containing medium and streaked onto plates containing 2% galactose to induce transcription of the mutated protein. Plates were incubated at the temperatures indicated for 3–4 days. Strain *DHD5* was used as a host in this experiment. The construction of the mutant is described in Materials and Methods. *pGALrho5\**, strain carrying the activated *RHO5Q91H* allele; wt, wildtype.

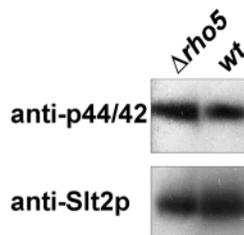
Fig. 5, in contrast to the *bem2*Δ single mutant, the *bem2 rho5* double mutant grows on rich medium at 25°C. The double mutant also grows at 30°C in the presence of up to 3 mM caffeine. However, as observed in the phenotypic analysis of *slt2 rho5* and *bck1 rho5* double deletions, the *bem2 rho5* strain still fails to grow at 37°C. This again suggests an additional function for Rho5p at 37°C apart from downregulation of the Pkc1p pathway.

#### Defects in regulation of actin dynamics in strains with *rho5* deletions and *RHO5Q91H* overexpression

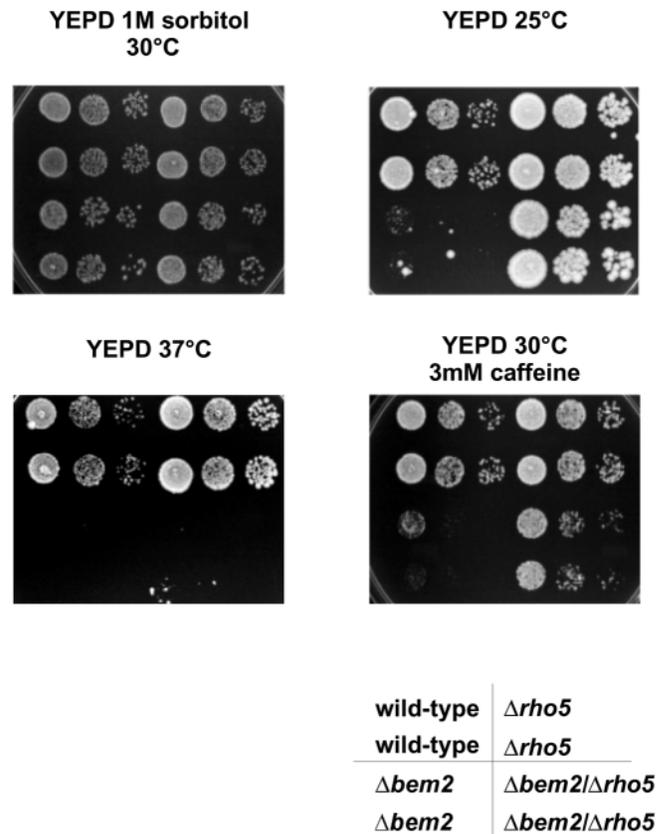
The proposed function of Rho5p as a negative downstream switch might be related to events that are regulated by two different branches of the same signalling pathway. Such a branched function in signalling has been suggested for Pkc1p. This kinase has been reported to be involved in the control of both depolarisation and repolarisation of the actin cytoskeleton upon heat shock (Delley and Hall, 1999). To see whether the negative regulatory function of Rho5p on the Slt2p MAPK



**Fig. 3.** Phenotypes of *bck1/rho5*- and *slt2/rho5*-double mutants. (A) Strains HPY1-2A ( $\Delta bck1$ ), HPY1-2D ( $\Delta rho5$ ), HPY1-2C ( $\Delta bck1, \Delta rho5$ ) and HPY1-2B (wt) were streaked out on rich medium and incubated at the temperatures shown and with the drugs indicated for 3-4 days. (B) Serial dilutions ( $10^3, 10^2, 10$ , from left to right) of strains HSH1-2B (wt), HSH1-4A ( $\Delta rho5$ ), HSH1-7B ( $\Delta slt2$ ) and HSH1-1A ( $\Delta slt2, \Delta rho5$ ) were spotted onto the media indicated and incubated at the given temperatures for 2 days.



**Fig. 4.** Slt2p phosphorylation in wild-type and  $\Delta rho5$  cells under inducing conditions at 37°C. 50  $\mu$ g of total protein was loaded in each lane and immunological detection was performed as described previously (Lorberg et al., 2001). Phosphospecific antibodies were used to detect the dually phosphorylated kinase in the upper lane. In the lower lane a polyclonal antiserum was employed as a loading control.



**Fig. 5.** Phenotypes of a *bem2/rho5* double mutant. Serial dilutions ( $10^3, 10^2, 10$ , from left to right) of strains HSH2-1B ( $\Delta rho5$ ), HSH2-5A (wt), HSH2-8B ( $\Delta bem2, \Delta rho5$ ) and HSH2-2B ( $\Delta bem2$ ) were incubated on full medium at the temperature and with the drugs indicated for 2 days.

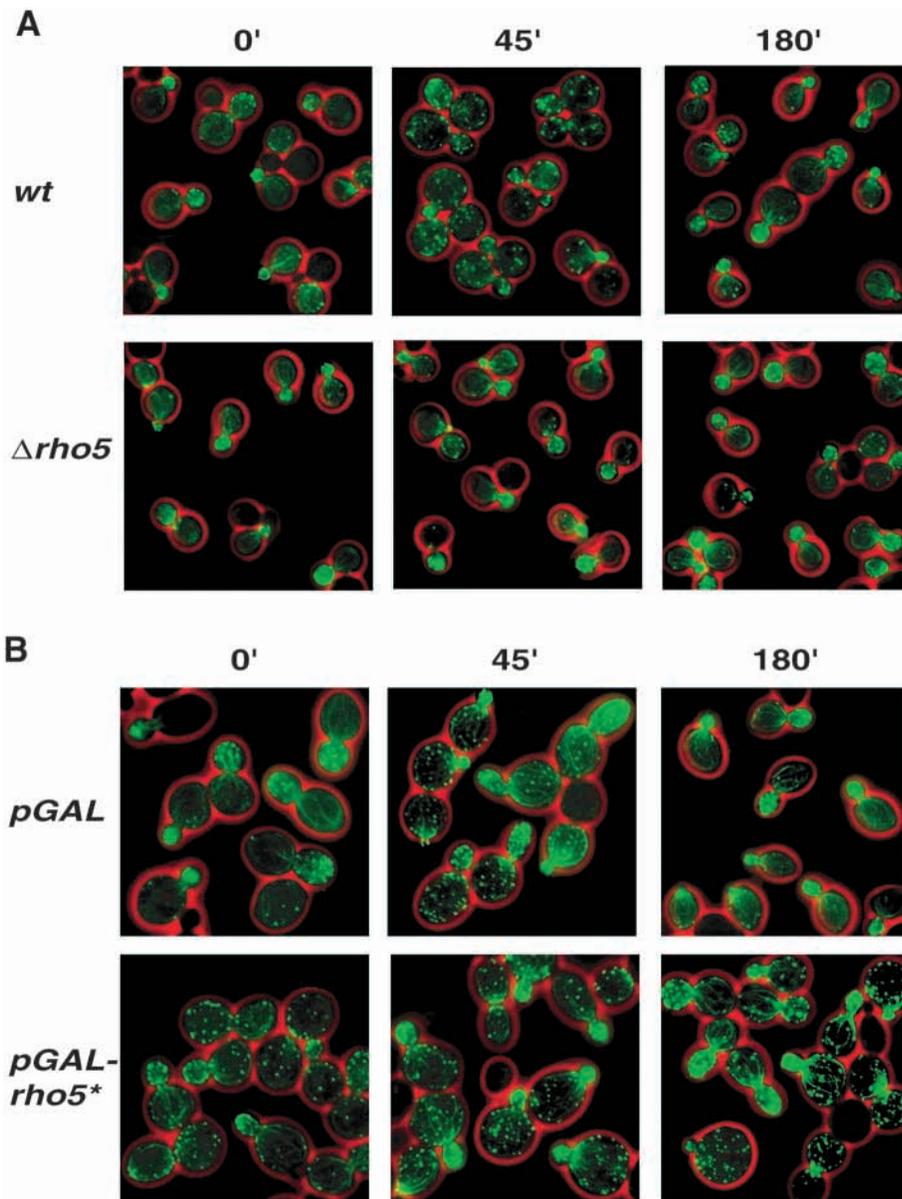
branch is involved in regulation of actin cytoskeleton polarity, we followed the distribution of actin in both the *rho5* deletion and in a strain overexpressing the activated *RHO5Q91H* allele (Fig. 6). Whereas wild-type cells showed depolarisation after 45 minutes at 37°C, the *rho5* deletion strain displayed no significant changes in actin polarity (Fig. 6A). In contrast, the activated *RHO5Q91H* mutant had its actin depolarised even before the shift to the higher temperature (i.e. at time 0; Fig. 6B). This depolarisation persisted even after 180 minutes. In the latter cells another consequence of the downregulation of the cellular integrity pathway becomes apparent after 180 minutes: cells expressing the activated allele increase in size, an observation also reported for *pkc1* mutants (Paravicini et al., 1992).

## Discussion

In this work we attempted to assign a biological function to the small G protein Rho5p in *S. cerevisiae*. The investigation was prompted by the observation that a *rho5* deletion led to an increase in the signal of the cellular integrity pathway mediated by the sole yeast homologue of mammalian protein kinase C. The increase in activity was observed in a routine indirect reporter assay employing a *lexA-Rlm1p* fusion protein coupled to the expression of the bacterial *lacZ* gene, and suggested a

negative regulatory function for Rho5p. Consistent with this hypothesis, we found that a *rho5* deletion is resistant to higher concentrations of caffeine (a compound against which pathway mutants are usually highly sensitive) than the wild-type. Conversely, the expression of an activated allele (*RHO5Q91H*) led to the expected increase in sensitivity against caffeine and other compounds such as Calcofluor white and Congo red. Such transformants also failed to grow at 37°C. Thus, they mimicked the phenotypes reported in other mutants defective for the signal transduction cascade (for a review, see Heinisch et al., 1999).

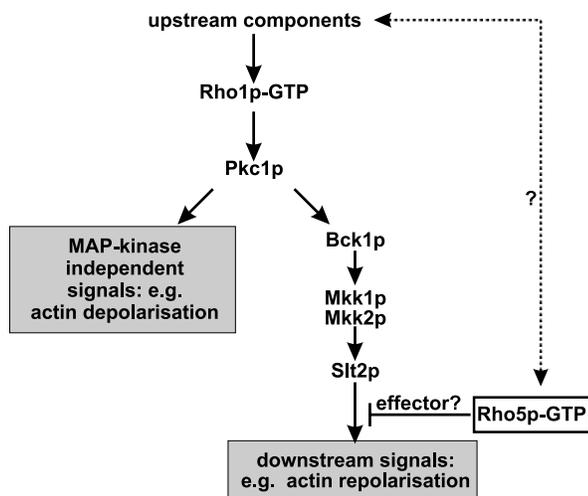
In order to determine the position of Rho5p within the signalling pathway we performed epistatic analyses with several different components. We showed that the caffeine-sensitivity of a *bck1* deletion, encoding the MAP kinase kinase of the pathway, was suppressed by an additional *rho5* deletion. Likewise, the growth defect of a *slt2* deletion strain (i.e. lacking the MAP kinase) in the absence of osmotic stabilization was also suppressed by elimination of Rho5p. This can be interpreted in two different ways. The first possibility is that Rho5p acts downstream of Slt2. The second, more indirect possibility would place Rho5p action in a pathway parallel to the Pkc1p signal transduction, such as the SVG pathway (Lee and Elion, 1999). This SVG pathway, like the Pkc1p pathway, regulates cell wall integrity and complementation of mutations in either pathway by components of the other pathway has been shown. Both pathways are able to control the transcription of *FKS2*, encoding a subunit of the  $\beta$ -(1,3)-glucan synthase, a component important for the integrity of the cell wall (Lee and Elion, 1999). But in contrast to the Pkc1p pathway, which controls *FKS2* transcription via Rlm1p, *FKS2* transcription relies on Ste12p and Tec1 in the SVG pathway. Because the reporter assay used in this study is based on changes in Rlm1p activation, an involvement of Rho5p in the SVG pathway is unlikely. It therefore is reasonable to place Rho5p action downstream of the MAP kinase cascade as depicted in Fig. 7. However, the temperature-sensitivity of *bck1*- and *slt2*-deletions was not alleviated by the *rho5* deletion. Rather, the sensitivity of *bck1* strains was exacerbated. This phenotype could be explained by the need for a highly balanced signal under heat stress



**Fig. 6.** Influence of Rho5p on actin dynamics. (A) Effect of a *rho5* deletion on the distribution of actin upon heat shock. Cells were investigated at the times indicated (for details, see Materials and Methods). Strains employed were HSH1-1C and HSH1-4A. (B) Effect of *RHO5Q91H* overexpression on actin kinetics. Strain DHD5 was transformed with pJH447 (vector not carrying any *RHO5* sequences) or pSH8 (pGAL*rho5*\*; vector carrying the activated *RHO5Q91H* allele). Yeast cells were grown in glucose-containing minimal medium overnight. Then cells were inoculated into 50 ml of fresh minimal medium containing the carbon source indicated and grown for about 4 hours to an optical density of between 0.3 and 0.6 at 30°C. To observe actin dynamics under heat shock the cultures were shifted to 37°C, and actin distribution after heat shock was investigated at the times indicated using the staining procedure described in Materials and Methods.

conditions. As observed in the regulation of actin dynamics, first an up- and then a downregulation would be required. Deletion of a gene encoding a negative regulatory element (i.e. Rho5p) could result in an imbalance interfering with the ability of the cell to cope with this specific stress condition.

The inhibitory function of Rho5p downstream of the MAP kinase Slt2p in the signal transduction pathway is also



**Fig. 7.** Model for Rho5p action. The possible action of Rho5p in the cellular integrity pathway consistent with the results reported in this work is indicated. For details, see Discussion.

consistent with our observation that Slt2p phosphorylation remains unaffected by a *rho5* deletion.

What is the possible advantage of such a downstream switch for the cell? In view of the branched nature of the Pkc1p-mediated signal transduction pathway (Lee et al., 1993a; Li et al., 2000) one can imagine that such a switch could turn off the signalling within one branch while leaving the other unaffected. For the cellular integrity pathway several such upstream signals that do not require an active MAP-kinase branch have been reported (see also citations in the Introduction). Among these upstream signals, some are independent of the signal generated by the MAP kinase and indeed require a downregulation of the cascade. Thus the depolarisation of actin is activated by Pkc1p independently of the MAP-kinase cascade, whereas the repolarisation requires the MAP kinase signal (Delley and Hall, 1999). Therefore, we also tested whether Rho5p is involved in this regulation. Consistent with the results reported above a deletion mutant of *rho5* showed a strongly reduced actin depolarisation after the shift to 37°C. However, overexpression of an activated *RHO5Q91H* allele led to a depolarisation already in the absence of heat stress. Apparently, in the *rho5* deletion the signal for repolarisation is permanently active (i.e. it cannot be switched off) and the actin cytoskeleton stays polarized. In contrast, overexpression of *RHO5Q91H* blocks the repolarisation signal by switching it off continuously and the actin cytoskeleton remains mainly depolarized.

Small GTPases are generally regulated by proteins that include GAPs, GEFs and GDIs (Mackay and Hall, 1998). As stated in the Introduction, for Rho5p only Rga2p has been identified as a likely GAP function (Roumanie et al., 2001). Using the phenotypes described herein will aid to the identification of more regulators of this protein. A first hint to such a regulator is provided by the suppression of *bem2* deletion phenotypes by a *rho5* deletion. Previous work indicated that Bem2p acts as a GAP for Rho1p as it shows GAP activity against it in vitro (Peterson et al., 1994). Additionally the deletion of *bem2* leads to an increased phosphorylation of Slt2p (Martin et al., 2000). However, the phenotypes of *bem2*

mutants are inconsistent with it acting primarily on Rho1p. Thus, cells are more sensitive to cell wall destabilizing drugs than would be expected. This increased sensitivity cannot be suppressed by overexpression of *SLT2* (Cid et al., 1998). Also *bem2* mutants display a depolarized actin cytoskeleton (Cid et al., 1998), again a phenotype not consistent with an activated MAP kinase pathway. Taken together these different results suggest a downregulation of the increased pathway activity downstream of Mpk1p/Slt2p. Because we could suppress some of the growth defects of a *bem2* deletion by additional deletion of *rho5* it seems likely that Rho5p is responsible for this downstream block. Consequently, Rho1p and Rho5p would share some of the regulatory components of the cellular integrity pathway with the possibility of a negative feedback loop. This model would explain the contradictory results for Bem2p described above.

In summary, the data presented here provide the first hints as to the biological function of Rho5p in *S. cerevisiae*. This negative switch apparently acts downstream of the MAP-kinase Mpk1p/Slt2p and is important for the regulation of events that require activated upstream components independently of the signal of the MAP-kinase cascade via Rlm1p itself (e.g. the regulation of actin polarisation and depolarisation under heat stress). Further work to identify other components of this negative regulatory pathway is in progress.

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