

# A synthetic analysis of the *Saccharomyces cerevisiae* stress sensor Mid2p, and identification of a Mid2p-interacting protein, Zeo1p, that modulates the *PKC1–MPK1* cell integrity pathway

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Mid2p is a plasma membrane protein that functions in *Saccharomyces cerevisiae* as a sensor of cell wall stress, activating the *PKC1–MPK1* cell integrity pathway via the small GTPase Rho1p during exposure to mating pheromone, calcofluor white, and heat. To examine Mid2p signalling, a global synthetic interaction analysis of a *mid2* mutant was performed; this identified 11 interacting genes. These include *WSC1* and *ROM2*, upstream elements in cell integrity pathway signalling, and *FKS1* and *SMI1*, required for 1,3- $\beta$ -glucan synthesis. These synthetic interactions indicate that the Wsc1p sensor acts through Rom2p to activate the Fks1p glucan synthase in a Mid2p-independent way. To further explore Mid2p signalling a two-hybrid screen was done using the cytoplasmic tail of Mid2p; this identified *ZEO1* (*YOL109w*), encoding a 12 kDa peripheral membrane protein that localizes to the plasma membrane. Disruption of *ZEO1* leads to resistance to calcofluor white and to a Mid2p-dependent constitutive phosphorylation of Mpk1p, supporting a role for Zeo1p in the cell integrity pathway. Consistent with this, *zeo1*-deficient cells suppress the growth defect of mutants in the Rho1p GDP–GTP exchange factor Rom2p, while exacerbating the growth defect of *sac7 $\Delta$*  mutants at 37 °C. In contrast, *mid2 $\Delta$*  mutants have opposing effects to *zeo1 $\Delta$*  mutants, being synthetically lethal with *rom2 $\Delta$* , and suppressing an 18 °C growth defect of *sac7 $\Delta$* , while overexpression of *MID2* rescues a *rom2 $\Delta$*  37 °C growth defect. Thus, *MID2* and *ZEO1* appear to play reciprocal roles in the modulation of the yeast *PKC1–MPK1* cell integrity pathway.

## INTRODUCTION

The cell wall of *Saccharomyces cerevisiae* is a complex and dynamic structure, essential for the maintenance of cell shape and integrity. Composed of 1,3- $\beta$ -glucan, 1,6- $\beta$ -glucan, mannoproteins and chitin, the cell wall undergoes changes in shape and composition during the vegetative budding cycle, as well as in the alternative developmental pathways of mating and sporulation. The integrity of the cell wall during these dynamic periods requires *PKC1*, a gene encoding the budding yeast homologue of mammalian protein kinase C, with mutants displaying a cell lysis defect (Levin & Bartlett-Heubusch, 1992). Cells lacking functional Pkc1p require an osmotic stabilizer for growth, and are sensitive to caffeine and high temperature. When shifted to a hypo-osmotic medium, *pkc1 $\Delta$*  mutants are unable to maintain cell wall integrity, and arrest growth with a small bud that lyses at its tip. Although the role of Pkc1p appears to be the activation of the Mpk1p MAP kinase cascade, it is also responsible for a less well characterized secondary

pathway co-ordinating ribosome and membrane synthesis (Nierras & Warner, 1999).

The Mpk1p MAP kinase cascade begins with the Pkc1p substrate, Bck1p (Costigan *et al.*, 1992; Lee & Levin, 1992). *BCK1* encodes a protein of the MAPKK kinase family. In response to activation by Pkc1p, Bck1p phosphorylates a pair of redundant MAPK kinases Mkk1p/Mkk2p (Irie *et al.*, 1993). In turn, these MAPK kinases dually phosphorylate the MAP kinase Mpk1p/Slt2p on tyrosine and threonine residues (Lee *et al.*, 1993). Phosphorylation of Mpk1p has been demonstrated to occur in response to high temperature, exposure to exogenous mating pheromone, or hypo-osmotic shock (Martin *et al.*, 1993; Buehrer & Errede, 1997; Zarzov *et al.*, 1996). Loss of *PKC1* function, or that of any of the components of the MAP kinase cascade under its control, results in a cell lysis defect. This defect is attributable, at least in part, to a defect in the transcription of a variety of genes involved in cell wall biosynthesis (Igual *et al.*, 1996). Regulation of the *PKC1–MPK1* cell integrity pathway is dependent on the Rho1p GTPase. GTP-bound Rho1p has been shown to physically associate with Pkc1p,

Abbreviation: HA, haemagglutinin.

resulting in its activation (Nonaka *et al.*, 1995; Kamada *et al.*, 1996). Essential for viability in *S. cerevisiae*, Rho1p localizes to sites of polarized growth and is implicated in cytoskeletal organization (Evangelista *et al.*, 1997). In addition to its role in the cell integrity pathway, Rho1p in its GTP bound form activates the 1,3- $\beta$ -glucan synthase complex through Fks1p (Qadota *et al.*, 1996). Several regulators of Rho1 activity have been identified: Bem2p (Peterson *et al.*, 1994) and Sac7p (Schmidt *et al.*, 1997) are GTPase activating proteins, while Rom1p and Rom2p act as GDP-GTP exchange factors for Rho1p (Ozaki *et al.*, 1996).

In a body of work, links have been made between the cell surface and the *PKC1-MPK1* cell integrity pathway (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998; Ketela *et al.*, 1999; Rajavel *et al.*, 1999; Philip & Levin, 2001; Sekiya-Kawasaki *et al.*, 2002). Acting as sensors for the cell wall, a number of type I membrane proteins respond to vegetative and stress-related growth requirements by activating the *PKC1-MPK1* cell integrity pathway. Two families of cell-surface sensors have been described. The first, the *WSC* family of four related genes, appears to be necessary for vegetative growth. The archetype member Wsc1p functions upstream of the cell integrity pathway, with *wsc1 $\Delta$*  mutants showing defective *PKC* pathway phenotypes. Like Wsc1p, both Mid2p and its functional homologue, Mtl1p, appear to be upstream activators of the cell integrity pathway (Ketela *et al.*, 1999). Both Mid2p and Mtl1p show structural similarity to the *WSC* family, but show little amino acid sequence identity. All contain a single membrane-spanning domain, an N-terminal signal sequence, an extracellular domain rich in serine/threonine residues, and a short cytoplasmic tail. Identified as a gene necessary for survival upon exposure to mating pheromone (Ono *et al.*, 1994), Mid2p is a type I integral plasma membrane protein. Null mutants of *mid2* show normal vegetative growth, although some strains have a temperature-sensitive lysis defect (Rajavel *et al.*, 1999). In addition to the mating-induced death phenotype, *mid2 $\Delta$*  mutants are sensitive to caffeine, and resistant to the chitin-binding dye calcofluor white, while overexpression of *MID2* leads to an increase in chitin levels and calcofluor white hypersensitivity (Ketela *et al.*, 1999). Both Wsc1p and Mid2p are required for the activation of the cell integrity pathway (Gray *et al.*, 1997; Ketela *et al.*, 1999; Rajavel *et al.*, 1999), and physically interact with Rom2p and increase GEF activity toward Rho1p (Philip & Levin, 2001). Despite these common functions, recent work indicates that Wsc1p is involved in Rho1p-dependent activation of the glucan synthase component, Fks1p, while Mid2p is not (Sekiya-Kawasaki *et al.*, 2002).

To extend this idea, we performed a screen for genes synthetically interacting with *MID2*, and found that in the absence of Mid2p, components activated by Wsc1p and Rom2p are essential. To examine the signalling role of Mid2p, we performed a two-hybrid screen with the essential cytoplasmic tail of Mid2p, and identified *ZEO1* (*YOL109w*). We show that *ZEO1* encodes a 12 kDa protein that primarily

localizes to the cell periphery; that *zeo1 $\Delta$*  mutants are resistant to calcofluor white; and that Zeo1p is necessary for calcofluor white hypersensitivity on high-copy expression of *MID2*. We also provide genetic and biochemical evidence linking *MID2* and *ZEO1* with the *PKC1-MPK1* cell integrity pathway.

## METHODS

**Strains, plasmids and media.** The *S. cerevisiae* strains used in this study are listed in Table 1 and oligonucleotide sequences are listed in Table 2. Removal of the *MID2* cytoplasmic domain was accomplished by introducing *Bgl*II sites at residues 871 and 1177 via site-directed mutagenesis (Kunkel *et al.*, 1987). A 306 bp fragment was removed and the resulting *MID2* construct was religated. The two-hybrid bait plasmid (pEG202*MID2*<sub>CYT</sub>) was created by generating an *Eco*RI restriction site through the modification of TGTATC (residues 744–749 in the *MID2* ORF) to GAATCC via site-directed mutagenesis on *MID2* contained in pBSII KS(–) with primer 1. A *Bam*HI restriction site was inserted directly downstream of the stop codon using primer 2. A 330 bp *Eco*RI–*Bam*HI fragment containing the cytoplasmic domain of *MID2* was cloned into corresponding sites in pEG202 to generate an in-frame LexA–*MID2*<sub>CYT</sub> fusion. Two-hybrid bait constructs were confirmed by DNA sequencing (Applied Biosystems).

Specific internal fragments of the *MID2* cytoplasmic domain were amplified from BY4741 genomic DNA with Expand DNA polymerase (Boehringer Mannheim) using the primer pairs 3 and 4, 5 and 6, and 7 and 8. PCR fragments were digested with *Eco*RI and *Bam*HI and subcloned into pEG202. Fidelity of the amplified fragments was confirmed by DNA sequencing.

*ZEO1* was amplified from BY4741 genomic DNA using Expand DNA polymerase and the primer pair 9 and 10. A 2.5 kb fragment was excised with *Sac*II and *Xho*I and cloned into the corresponding sites on pRS316 and pRS426. Fidelity of the PCR was confirmed by DNA sequencing.

Zeo1p–HA was created by insertion of a single copy of the haemagglutinin (HA) epitope (YPTDVPDYA) directly upstream of the stop codon between residues 338 and 339 via site-directed mutagenesis (Kunkel *et al.*, 1987) on single-stranded *ZEO1* contained on pRS316. Correct insertion of the HA epitope was confirmed by DNA sequencing. A 2.5 kb *Sst*II–*Xho*I fragment containing *ZEO1*–HA was excised and cloned into pRS426. Zeo1p–HA was shown to be functional by the observation that pRS316*ZEO1*–HA could fully complement *ZEO1* in a *zeo1 $\Delta$  rom2 $\Delta$*  mutant.

**Synthetic lethal mutant screen.** Systematic genetic array analysis (SGA) was used to identify genes essential in a *mid2 $\Delta$*  background, as described by Tong *et al.* (2001). Briefly, HAB976 (Table 1) was obtained in four steps. First, the *mid2 $\Delta$ ::KanMX4* from YD5241 (Table 1) was switched to *mid2 $\Delta$ ::NatMX4* by PCR-based transformation. Second, the Nat<sup>R</sup> transformants were mated to Y3084 (Table 1) and the *MAT $\alpha$ / $\alpha$*  diploids were transferred onto sporulation medium. *MAT $\alpha$*  meiotic progeny were then selected on synthetic medium lacking leucine and arginine but containing canavanine. The mating type was confirmed by PCR, according to Huxley *et al.* (1990). Third, cells were replica plated onto medium containing nourseothricin to select for the deletion mutants. Fourth, cells were replica plated onto medium lacking lysine to identify *lys2 $\Delta$*  derivatives. From three SGA screens, 194 potential positives were identified; 11 were confirmed by tetrad analysis.

**Localization of Zeo1p.** A *ZEO1*–*GFP* fusion was generated by inserting a *Bam*HI and *Not*I site over the start codon via site-directed

**Table 1.** Description of strains used in this study

Strain	Genotype	Source
Y1003	<i>MAT<math>\alpha</math> URA3::lexAop-lacZ/lexAop-ADE2::URA3 ura3-1/ura3-1 leu2-3/leu2-3 his3-11/his3-11 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100</i>	C. Boone
SEY6210 diploid	<i>MAT<math>\alpha</math>/a leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-<math>\Delta</math>200/his3-<math>\Delta</math>200 lys2-801/lys2-801 trp1-<math>\Delta</math>901/trp1-<math>\Delta</math>901 suc2-<math>\Delta</math>9/suc2-<math>\Delta</math>9</i>	Ketela <i>et al.</i> (1999)
BY4741	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 met15<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Brachmann <i>et al.</i> (1998)
BY4742	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Brachmann <i>et al.</i> (1998)
BY4743	<i>MAT<math>\alpha</math>/a BY4741/BY4742</i>	Brachmann <i>et al.</i> (1998)
YD5241	BY4741 <i>mid2<math>\Delta</math>::KanMX4</i>	Winzeler <i>et al.</i> (1999)
YD20993	BY4743 <i>mpk1<math>\Delta</math>::KanMX4/MPK1</i>	Winzeler <i>et al.</i> (1999)
YD24225	BY4743 <i>sac7<math>\Delta</math>::KanMX4/SAC7</i>	Winzeler <i>et al.</i> (1999)
YD25241	BY4743 <i>mid2<math>\Delta</math>::KanMX4/MID2</i>	Winzeler <i>et al.</i> (1999)
YD25280	BY4743 <i>rom2<math>\Delta</math>::KanMX4/ROM2</i>	Winzeler <i>et al.</i> (1999)
YD26259	BY4743 <i>zeo1<math>\Delta</math>::KanMX4/ZEO1</i>	Winzeler <i>et al.</i> (1999)
HAB970	SEY6210 <i>rom2<math>\Delta</math>::KanMX4/ROM2 mid2<math>\Delta</math>::KanMX4/MID2</i>	This study
HAB971	BY4743 <i>zeo1<math>\Delta</math>::KanMX4/ZEO1 mid2<math>\Delta</math>::KanMX4/MID2</i>	This study
HAB972	BY4743 <i>sac7<math>\Delta</math>::KanMX4/SAC7 zeo1<math>\Delta</math>::KanMX4/ZEO1</i>	This study
HAB973	BY4743 <i>sac7<math>\Delta</math>::KanMX4/SAC7 mid2<math>\Delta</math>::KanMX4/MID2</i>	This study
HAB974	BY4743 <i>zeo1<math>\Delta</math>::KanMX4/ZEO1 rom2<math>\Delta</math>::KanMX4/ROM2</i>	This study
HAB975	SEY6210 <i>sac7<math>\Delta</math>::KanMX4/SAC7 mid2<math>\Delta</math>::KanMX4/MID2</i>	This study
HAB976	<i>MAT<math>\alpha</math> mid2<math>\Delta</math>::NatMX4 mfx1<math>\Delta</math>::MF<math>\alpha</math>1-prLEU2 can1<math>\Delta</math>::MFA1-prHIS3 his3<math>\Delta</math> leu2<math>\Delta</math> lys2<math>\Delta</math> met15<math>\Delta</math> ura3<math>\Delta</math></i>	This study
Y3084	<i>MAT<math>\alpha</math> mfa1<math>\Delta</math>::MF<math>\alpha</math>1pr-LEU2 can1<math>\Delta</math>::MFA1pr-HIS3 his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math></i>	Tong <i>et al.</i> (2001)

mutagenesis (Kunkel *et al.*, 1987) with primer 11, using single-stranded ZEO1 contained on pRS316 as template. Clones positive for incorporation of the restriction sites were confirmed by DNA sequencing. Generation of an in-frame GFP-ZEO1 fusion (GFP F64L S65T; kindly provided by U. Stochaj) was accomplished via a GFP BamHI-BamHI and ZEO1 BamHI-BamHI ligation. Correct orientation of the GFP construct was verified with a *NotI* diagnostic digestion. Functionality of the GFP-ZEO1 construct was demonstrated as described for ZEO1-HA. Localization of GFP-ZEO1 was accomplished by examination of live mid-exponential-phase cells carrying pRS316 GFP-ZEO1.

**Calcofluor white tests.** To test strains for sensitivity to calcofluor white, mid-exponential-phase cells were diluted and spotted either onto YEPD agar plates with the indicated amount of calcofluor white, or onto selective media buffered with 10 g MES l<sup>-1</sup> and adjusted to pH 6.2. Plates were incubated in the dark.

**Chitin assay.** Total cellular chitin was measured as described by Bulawa *et al.* (1986), and outlined by Ketela *et al.* (1999). In brief, washed cells (approx. 25 mg wet cells) were resuspended in 500  $\mu$ l 6% (v/v) potassium hydroxide and incubated at 80 °C for 90 min. After cooling at room temperature, 50  $\mu$ l glacial acetic acid was added. Insoluble material was washed twice with water and resuspended in 250  $\mu$ l 50 mM sodium phosphate (pH 6.3); 2 mg *Streptomyces griseus* chitinase (Sigma) was added, and incubated at 25 °C for 2 h. Tubes were centrifuged at 15 000 g for 5 min; 250  $\mu$ l of the supernatant was transferred to a fresh tube and 1 mg *Helix pomatia*  $\beta$ -glucuronidase (Sigma) was added. Tubes were incubated at 37 °C for 2 h and then assayed for N-acetylglucosamine content.

**Western blot and solubilization tests.** For solubilization tests, total cell extracts were prepared from mid-exponential-phase cells grown in selective medium, by vigorous vortexing in lysis buffer [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 5% (v/v) glycerol] in the presence of glass beads and protease inhibitors (Complete protease

inhibitor cocktail; Boehringer Mannheim). The resulting slurry was clarified by centrifugation at 3500 g for 10 min at 4 °C to remove unbroken cells and cell walls. The resulting supernatant was divided and one aliquot subjected to centrifugation at 50 000 g at 4 °C for 30 min. The supernatant was withdrawn, and the pellet resuspended in an equal volume of lysis buffer. Samples were resolved by SDS-PAGE and then subjected to Western blotting. Immunodetection of Zeo1p-HA was achieved by using anti-HA monoclonal antibody HA11 (Babco) at 1:1000 dilution and horseradish-peroxidase-conjugated secondary antibody (Amersham Life Sciences) at a 1:2000 dilution. Bands were visualized by enhanced chemiluminescence (Amersham Life Sciences).

**Measurement of Mpk1p phosphorylation.** The detection of Mpk1p/Slt2p phosphorylation was carried out as described by Martin *et al.* (2000). In brief, cells were grown overnight in selective medium at room temperature. For heat-shocked cells, overnight cultures were diluted and grown for 3 h at 37 °C. A 25 ml sample of mid-exponential-phase cells was collected in an equal volume of ice-cold water and centrifuged at 4 °C for 4 min at 1623 g. The cell pellet was briefly washed in 1 ml ice-cold water and transferred to a pre-chilled 1.5 ml collection tube. Cells were repelleted for 20 s at 15 000 g. The supernatant was immediately removed and the pellet resuspended in 300  $\mu$ l ice-cold lysis buffer [50 mM Tris/HCl pH 7.5, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM  $\beta$ -glycerol phosphate, 5 mM sodium pyrophosphate], in the presence of glass beads and protease inhibitors (Complete protease inhibitor cocktail; Boehringer Mannheim). Equal concentrations of protein were fractionated by 8% (v/v) SDS-PAGE. Membranes were probed with phospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody (New England Biolabs) at 1:500 dilution to detect dually phosphorylated Mpk1p. Control of sample loading was monitored using anti-HA, as described above.

**Table 2.** Oligonucleotide primers used in this study

No.	Description/use	Sequence
1	Generation of <i>MID2</i> two-hybrid bait (5')	TTAAATTACATGTTTGAATTCCAAATCATCAAGGACG
2	Generation of <i>MID2</i> two-hybrid bait (3')	TCACACGAAATTAATAAGGGATCTCATATATCCATTCATATCA
3	<i>MID2</i> <sub>CYT</sub> aa250–313 <i>EcoRI</i>	GAGCTCGAATTCCTCAATCATCAAGGACGGAC
4	<i>MID2</i> <sub>CYT</sub> aa250–313 <i>BamHI</i>	GAGCTCGGATCCACACATCATCTGCTTCATCTAG
5	<i>MID2</i> <sub>CYT</sub> aa314–376 <i>EcoRI</i>	GAGCTCGAATTCGTGATGGAGCAAAAGCTCC
6	<i>MID2</i> <sub>CYT</sub> aa314–376 <i>BamHI</i>	GAGCTCGGATCCTTAATAATTTCTGGTIG
7	<i>MID2</i> <sub>CYT</sub> aa282–343 <i>EcoRI</i>	GAGCTCGAATTCGGTAAGAAAAGTTAGTGATG
8	<i>MID2</i> <sub>CYT</sub> aa282–343 <i>BamHI</i>	GAGCTCGGATCCTCCACCCGCTATTTGGTATTATATG
9	Amplification of <i>ZEO1</i> (5')	TTTCCCGCGGACAGACGACGACGGAAGACC
10	Amplification of <i>ZEO1</i> (3')	TTTCCGCTGAGACTAATCATATACCTGAAAAGC
11	<i>ZEO1</i> N-term. GFP	CTACGTTTATATCAATTAATAGGATCCGAGCTCGGGCCGCACTCTGAAATTCAAAACAAAAGCTG

## RESULTS

### Synthetic interaction analysis of *MID2*

To broadly assess Mid2p function, we made a systematic genetic analysis (Tong *et al.*, 2001) on a *mid2* deletion mutant, globally examining the synthetic interactions of *MID2* with mutants in all nonessential genes. Previous work (Ketela *et al.*, 1999) identified synthetic lethal interactions in two genes, *WSC1* and *FKS1*. Under the vegetative growth conditions tested we found these, and an additional set of nine genes which when individually deleted led to a synthetic growth defect in a *mid2* deletion background (Table 3). Seven of these genes are involved in aspects of the cell wall synthesis or in the PKC1-dependent cell integrity pathway response. Of these, *FKS1*, *CCW12*, *VAN1* and *FPS1* encode a 1,3- $\beta$ -glucan synthase component, a structural cell wall component, a mannosyltransferase and a glycerol transporter required for normal response to osmotic stress, respectively (Douglas *et al.*, 1994; Luyten *et al.*, 1995; Mrsa *et al.*, 1997; Jungmann *et al.*, 1999). Individual deletion of these genes leads to cell surface stress. Mid2p is required for survival of these mutants, probably by activating the *PKC1*–*MPK1* cell integrity pathway (Ketela *et al.*, 1999; Philip & Levin, 2001). Concordant with this explanation are the known synthetic lethal interactions between components of the PKC pathway with *FKS1* and *FPS1* (Garret-Engle *et al.*, 1995; Tamas *et al.*, 1999) and transcription of *CCW12* being Mpk1p-dependent (Baetz *et al.*, 2001). Furthermore, three genes (*WSC1*/*SLG1*, *ROM2* and *SMI1*/*KNR4*) that act to maintain normal activity of Fks1p also show synthetic interactions with *MID2*. *WSC1* and *ROM2* encode upstream activators of the cell integrity pathway. Wsc1p is a membrane sensor and Rom2p a GDP–GTP exchange factor for Rho1p. *WSC1* and *ROM2* signal through Rho1p which, in turn, both activates the PKC pathway and stimulates 1,3- $\beta$ -glucan synthesis by interacting directly with Fks1p (Qadota *et al.*, 1996; Sekiya-Kawasaki *et al.*, 2002). *SMI1* is a positive regulator of glucan synthase activity, and shows genetic interactions with components of the PKC pathway (Martin-Yken *et al.*, 2002)

Our results are consistent with cell wall assembly being regulated by two distinct networks involving Rho1p (Sekiya-Kawasaki *et al.*, 2002). One involves signalling from Mid2p through Rho1p to Pkc1p and the *PKC1*–*MPK1* pathway, with the second involving specific signalling from Wsc1p and Rom2p through Rho1p to activate Fks1p, as well as to activate the *PKC1*–*MPK1* pathway. A prediction from this idea is that the effects of a *MID2* deletion should be confined to the *PKC1*–*MPK1* pathway. To test this we explicitly examined for synthetic effects of a *mid2* deletion with mutants in *bck1* and *mpk1*, downstream components of the *PKC1*–*MPK1* pathway. Neither *bck1mid2* nor *mpk1mid2* double mutants showed synthetic effects. Thus, absence of the Wsc1p/Rom2p/Fks1p network is required for synthetic lethality with a *mid2* deletion. Of the remaining genes interacting with *MID2*, *SAC6* and

**Table 3.** Synthetic interactions with *MID2*

Cellular role	Gene	ORF	YPD comment
Cell wall maintenance	<i>FKS1</i>	<i>YLR342W</i>	Component of 1,3- $\beta$ -glucan synthase
	<i>CCW12</i>	<i>YLR110C</i>	Cell wall mannoprotein
	<i>FPS1</i>	<i>YLL043W</i>	Glycerol channel protein
	<i>WSC1</i>	<i>YOR008C</i>	Plasma membrane protein required for maintenance of cell wall integrity and for the stress response during vegetative growth
	<i>ROM2</i>	<i>YLR371W</i>	GDP-GTP exchange factor for Rho1p
	<i>SMI1</i>	<i>YGR229C</i>	1,3- $\beta$ -Glucan synthesis regulatory protein
Other	<i>VAN1</i>	<i>YML115C</i>	Vanadate-resistance protein; mannosyltransferase, component of the mannan-Pol I
	<i>SAC6</i>	<i>YDR129C</i>	Actin filament bundling protein, fimbrin
	<i>ILM1</i>	<i>YJR118C</i>	Increased Loss of Mitochondrial DNA
		<i>YLR111W</i>	Hypothetical protein
		<i>YLR338W</i>	Questionable ORF; overlaps with <i>VRP1</i>

*YLR338w* (which overlaps with *VRP1*) are involved in polarity and cytoskeleton function, *ILM1* is required for mitochondrial inheritance, and *YLR111W* is of unknown function.

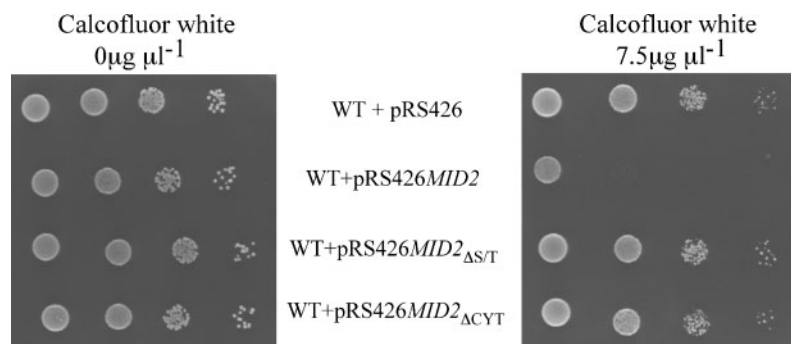
### The cytoplasmic tail of Mid2p is essential for function

As Mid2p shows signalling distinct from Wsc1p, we examined how this signalling specificity was mediated. To act as a stress sensor this plasma-membrane-spanning protein must be able both to detect stress and to relay this information intracellularly. The large highly O-mannosylated extracellular domain presumably involved in signal detection is essential for function (Ketela *et al.*, 1999; and see Fig. 1). To test if the cytoplasmic tail of Mid2p is required for this intracellular relay, we constructed a Mid2p mutant with a deletion of the C-terminal cytoplasmic domain (amino acid residues 252–376) from *MID2*, generating *MID2<sub>ΔCYT</sub>* under the control of the *MID2* promoter. Although this mutant localized normally to the cell periphery (not shown), it was unable to confer sensitivity to calcofluor white when expressed at multicopy levels (Fig. 1). Philip & Levin (2001) demonstrated that a deletion of this domain also fails to complement the mating-induced death phenotype of *mid2Δ* mutants. These results

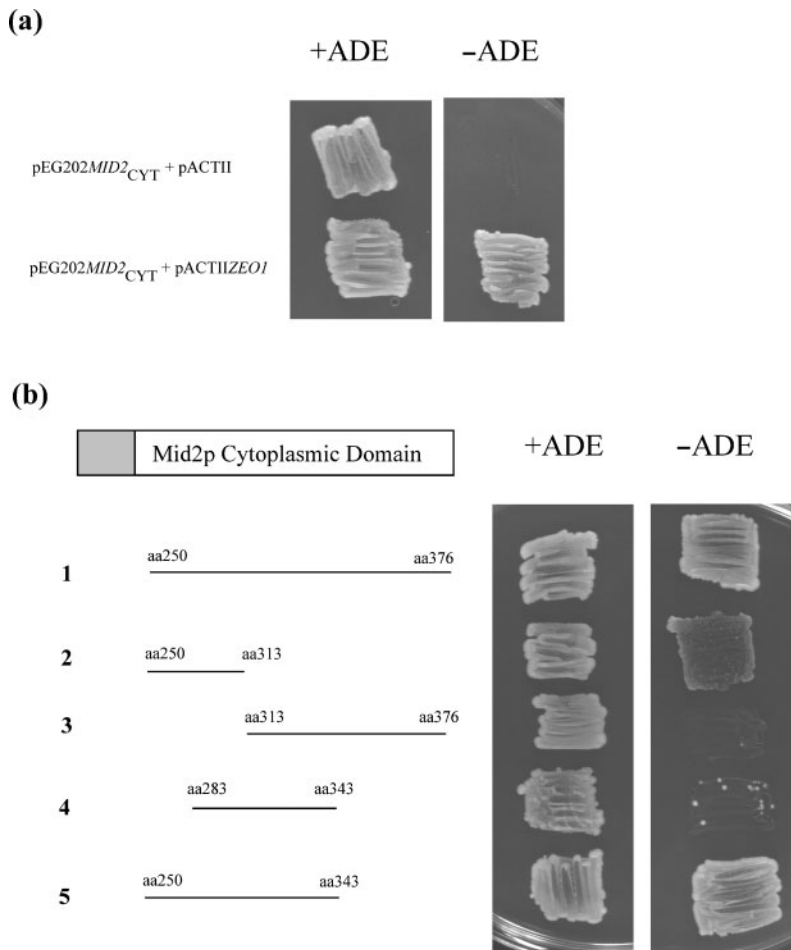
indicate that the cytoplasmic domain is required for a fully functional Mid2p.

### Zeo1p interacts with the cytoplasmic domain of Mid2p in a two-hybrid screen

To explore the role of the cytoplasmic domain in Mid2p function, a two-hybrid screen was made to identify physically interacting proteins. The sequence encoding the cytoplasmic domain of *MID2* (amino acid residues 250–376) was subcloned in-frame to the LexA DNA-binding domain of a two hybrid vector (pEG202-NLS; C. Boone). Sequence analysis showed that all clones were full-length and in-frame with the Gal4 activating domain of the pACTII bait plasmid. One clone containing the gene *ZEO1* (*YOL109w*) was identified several times in the screen and studied further (Fig. 2a). To map the Zeo1p-binding domain of the Mid2p tail, a variety of LexA-*MID2<sub>ΔCYT</sub>* fusions were made and tested for interaction with pACTII*ZEO1* (Fig. 2c). A shortened domain between amino acid residues 250–343 was sufficient for the interaction, while a fusion between residues 250–313 showed a weakened interaction. The polypeptide directly adjacent to the transmembrane domain is required for Mid2p interaction with Zeo1p, since cytoplasmic domain fusions that do not contain this



**Fig. 1.** The cytoplasmic and extracellular domains of Mid2p are essential for function. Mid-exponential-phase BY4742 cells (wild-type) containing vector only, multicopy *MID2*, multicopy *MID2<sub>ΔS/T</sub>*, or multicopy *MID2<sub>ΔCYT</sub>*, were diluted to a concentration of  $3 \times 10^6$  cells ml<sup>-1</sup>; 5 μl of this suspension and three subsequent 10-fold serial dilutions were each spotted onto medium containing the indicated concentration of calcofluor white.



**Fig. 2.** (a) The cytoplasmic domain of Mid2p interacts with Zeo1p in a two-hybrid system. W303 containing pEG202MID2<sub>CYT</sub> (aa250–376), with either vector alone (pACTII) or pACTIIZEO1, were streaked onto medium with or without adenine. (b) Mid2p interaction with Zeo1p is dependent on discrete portions of the cytoplasmic domain. W303 containing pEG202MID2<sub>CYT</sub> (aa250–376), pEG202MID2<sub>CYT</sub> (aa250–313), pEG202MID2<sub>CYT</sub> (aa313–376), pEG202MID2<sub>CYT</sub> (aa283–343), or pEG202MID2<sub>CYT</sub> (aa250–343) and pACTIIZEO1 were streaked on medium with or without adenine and incubated at 30 °C for 3 days.

region (amino acid residues 283–343 and 314–376; 3 and 4 respectively in Fig. 2b) showed no interaction.

### Characterization and subcellular localization of Zeo1p

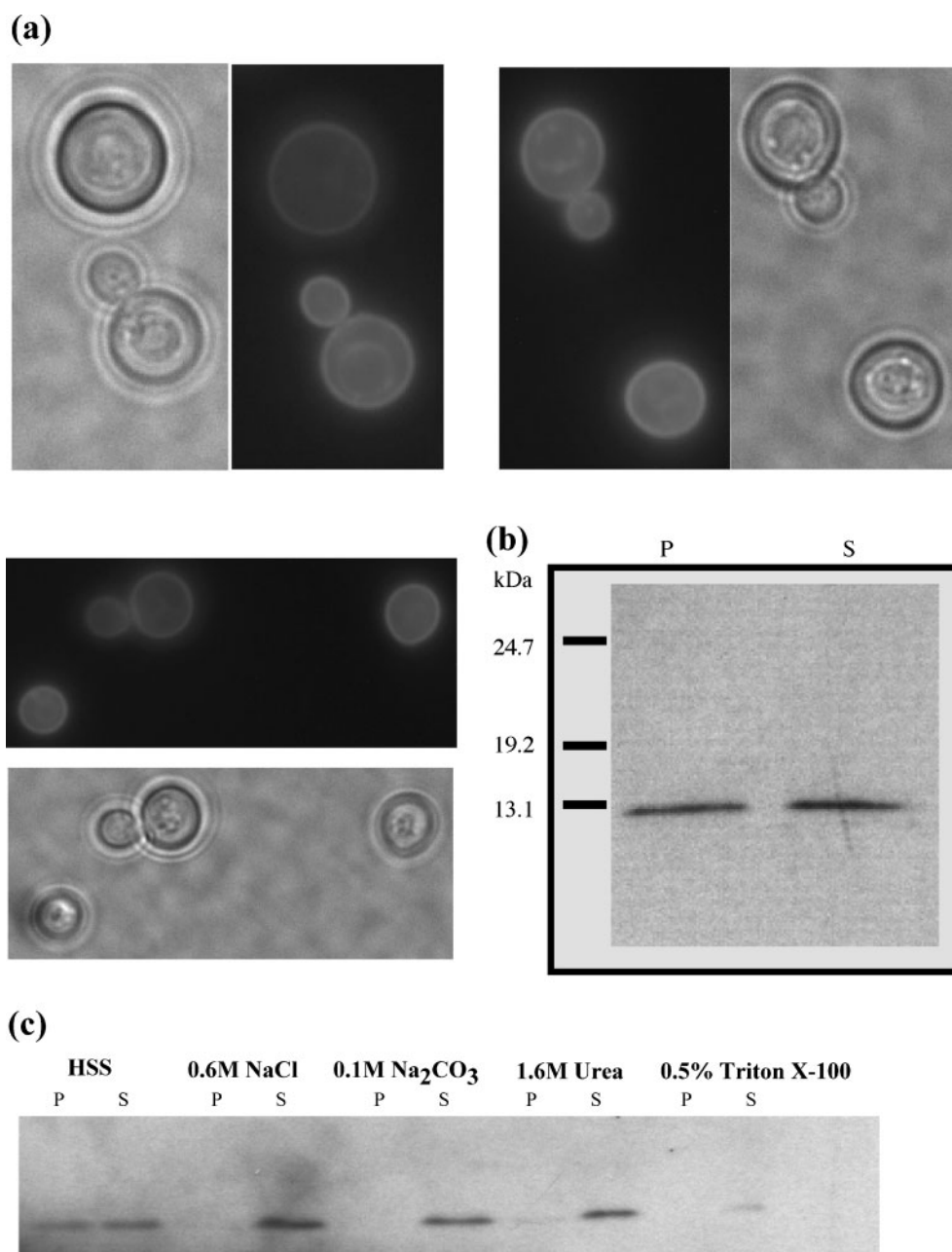
To examine the physical characteristics of Zeo1p, a functional HA-tagged protein, Zeo1p–HA, was generated which migrates at its predicted molecular mass of approximately 12 kDa (Fig. 3b). To test membrane association of Zeo1p, partially purified extracts from cells expressing Zeo1p–HA were fractionated into supernatant (soluble) and pellet (membrane-associated) portions by centrifugation. Zeo1p–HA was found in both the high-speed (HSS) (50 000 g) spin supernatant, as well as the pellet fraction (Fig. 3b). To test whether membrane association is peripheral or integral, partially purified, membrane-containing cell extracts were treated with urea, sodium chloride and sodium carbonate to disrupt peripheral or protein–protein associations, or with Triton X-100 to disrupt integral association. Zeo1p–HA was solubilized in all treatments, indicating that the insolubility is due to peripheral membrane and/or Zeo1p–membrane protein interactions (Fig. 3c).

Direct immunofluorescence microscopy was performed to establish the subcellular location of Zeo1p. A functional

Zeo1p–GFP fusion protein was constructed by inserting GFP immediately downstream of the ZEO1 start codon. Examination of the fluorescing cells maintaining centromeric Zeo1p–GFP revealed Zeo1p–GFP distribution to be mainly confined to the cell periphery (Fig. 3a). Since Zeo1p is a soluble protein with peripheral membrane association, it appears that this association is with the plasma membrane. Given the two-hybrid interaction with Mid2p, this localization could be, at least in part, due to association with Mid2p. However, examination of both *mid2Δ* and *mtl1Δ* cells maintaining centromeric Zeo1p–GFP showed the same peripheral pattern of staining (not shown).

### A *zeo1Δ* mutant is resistant to calcofluor white, and MID2-induced hypersensitivity to calcofluor white is Zeo1p-dependent

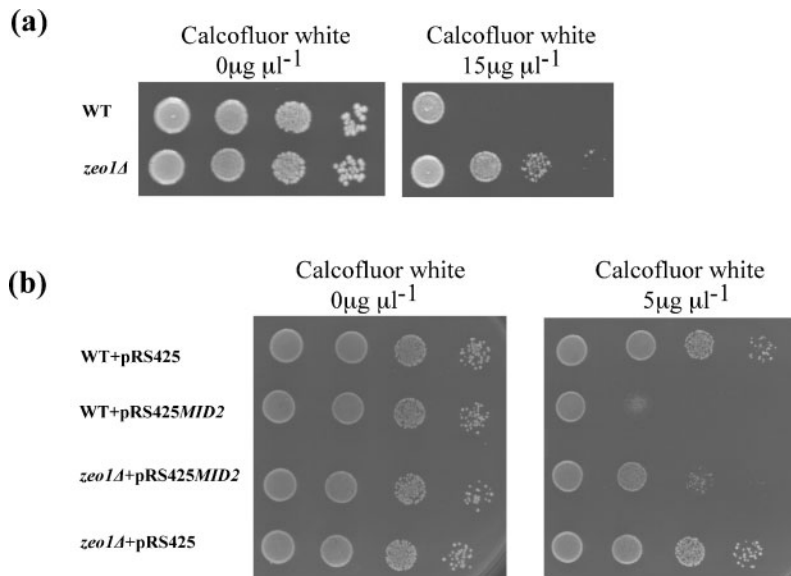
Chitin is vital to maintain cell wall integrity. Calcofluor white is a fluorescent dye that intercalates with nascent chitin chains, preventing microfibril assembly (Elorza *et al.*, 1983) and inhibiting growth. Previously, we demonstrated that *mid2Δ* cells are resistant to calcofluor white and that MID2 is required for synthesis of supplemental chitin under conditions of cell stress (Ketela *et al.*, 1999). If Zeo1p provides a structural link in Mid2p-mediated signalling, then *zeo1Δ* cells should also display a calcofluor white



**Fig. 3.** Cellular localization studies on Zeo1p. (a) GFP fluorescence analysis was performed on BY4742 (wild-type) cells expressing pRS316ZEO1-GFP. (b) Total cell extracts were centrifuged at 50 000 *g* and the resulting pellet (P) and soluble (S) fractions were analysed by Western blotting. (c) Immunoblot analysis of cell fractions from BY4742 containing pRS316ZEO1-HA to demonstrate membrane association. P, pellet; S, supernatant.

resistance phenotype. The results in Fig. 4(a) show that at a calcofluor white concentration of  $15 \mu\text{g ml}^{-1}$  on rich medium, *zeo1Δ* cells are more resistant to calcofluor white than wild-type cells. Cells harbouring *MID2* on a 2  $\mu\text{m}$  plasmid show enhanced sensitivity to calcofluor white, and we tested if *ZEO1* was involved in this hypersensitivity. Fig. 4(b) shows that *zeo1Δ* mutants overexpressing *MID2* were less sensitive to calcofluor white than a corresponding wild-type. This attenuation of hypersensitivity to calcofluor

white was partial, suggesting the involvement of additional proteins in this process. Resistance to calcofluor white is often associated with defects in chitin synthesis, and this is the case for *mid2Δ*-associated calcofluor white resistance and *MID2*-induced hypersensitivity (Ketela *et al.*, 1999). Overexpression of *MID2* causes hypersensitivity to calcofluor white by increasing total cell wall chitin levels (Ketela *et al.*, 1999). Since *zeo1Δ* mutants are able to attenuate *MID2*-mediated hypersensitivity to calcofluor white, we



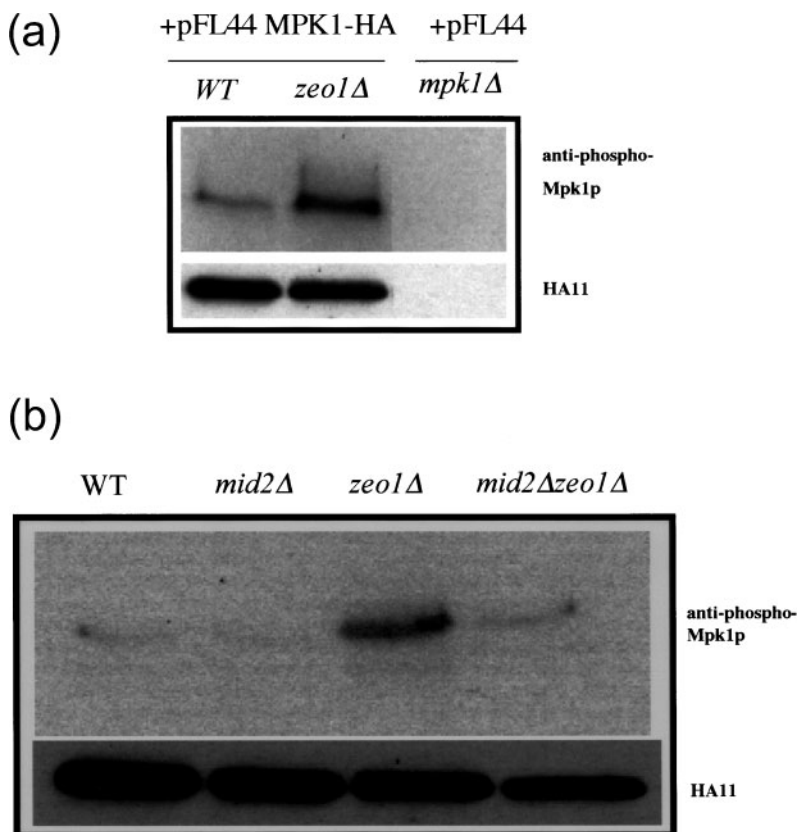
**Fig. 4.** *zeo1Δ* confers resistance to calcofluor white and is required for *MID2*-induced hypersensitivity. (a) Mid-exponential-phase cells were diluted to a concentration of  $3 \times 10^6$  cells  $\text{ml}^{-1}$ ; 5  $\mu\text{l}$  of this suspension and three subsequent tenfold serial dilutions were spotted onto YEPD plates containing the indicated concentration of calcofluor white and incubated at 30 °C for 3 days. (b) BY4742 cells (WT) containing pRS425 (vector alone) or pRS425MID2, and *zeo1Δ* cells containing pRS425MID2 or pRS425 (vector alone) were spotted in serial dilution on the indicated medium.

tested if this was through a reduction in the *MID2*-associated increase in chitin. However, we found that this increase in chitin was independent of *ZEO1*.

### Zeo1p affects basal activity of Mpk1p in a Mid2p-dependent manner

Mid2p is required for activation of the Mpk1 MAPK in response to stress (Ketela *et al.*, 1999; Rajavel *et al.*, 1999). To

determine if *ZEO1* is needed for this activation, we measured heat-induced activation of Mpk1p in a *zeo1Δ* strain. While heat-induced phosphorylation of Mpk1p was not reduced in a *zeo1Δ* strain (not shown), the basal level of Mpk1p phosphorylation was elevated in a *zeo1Δ* strain relative to wild-type at 22 °C (Fig. 5a). This result suggests a role for Zeo1p in the negative regulation of the cell integrity pathway. Given the relationship between Mid2p and Zeo1p, we asked if the *zeo1Δ* increase in basal activation of Mpk1p



**Fig. 5.** *zeo1Δ* mutants show a Mid2p-dependent increase in the basal level of phosphorylation of Mpk1p. (a) Lanes are loaded with equal concentrations of total cellular extracts from wild-type, *zeo1Δ* and *mpk1Δ*. (b) Total cellular extracts from wild-type (WT), *mid2Δ*, *zeo1Δ* and *mid2Δzeo1Δ*, all carrying pFL44Mpk1-HA. Mpk1p phosphorylation was detected by anti-phospho-44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) (New England Biolabs). Loading control for Mpk1-HA is demonstrated by anti-HA antibody HA11.

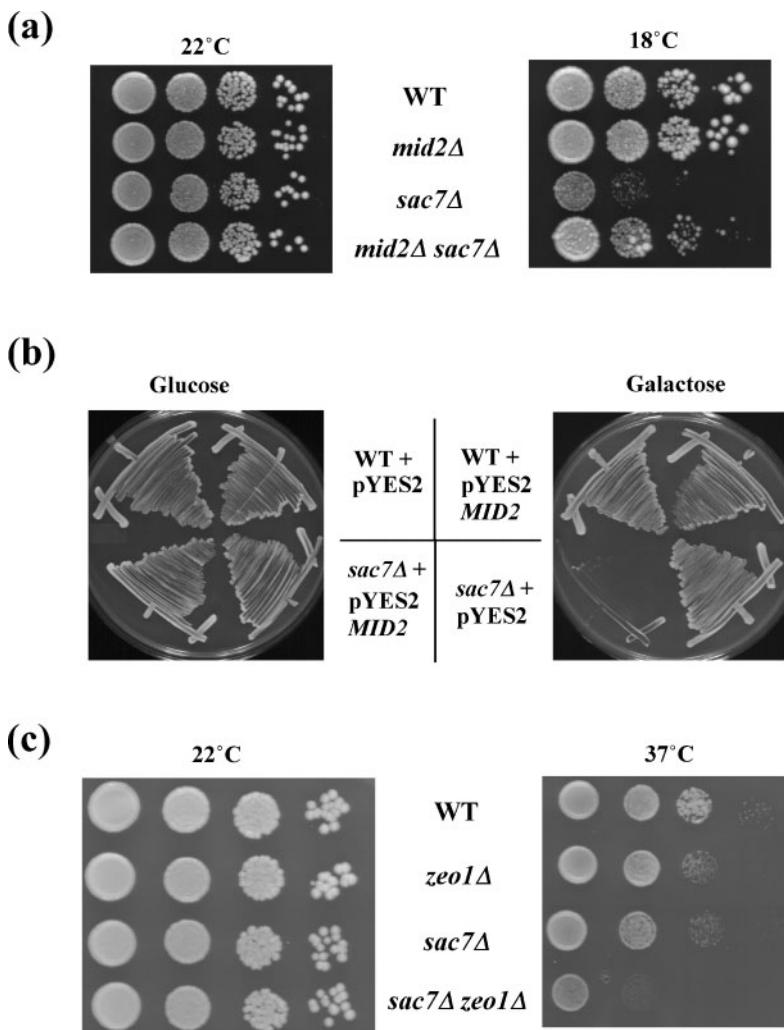
required Mid2p. As shown in Fig. 5(b), a *mid2Δzeo1Δ* mutant had basal Mpk1p activity, indicating the need for Mid2p in this activation.

### Mid2p and Zeo1p signal to the cell integrity pathway via the Rho1p GTPase

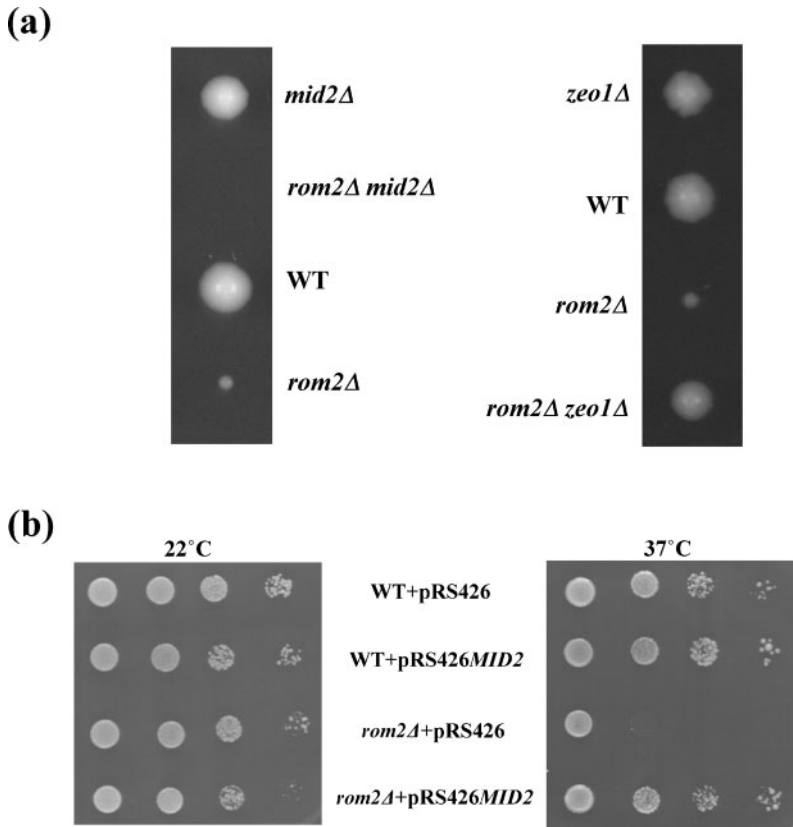
Mid2p acts on the Rho1p GTPase to activate the *PKC1-MPK1* cell integrity pathway (Ketela *et al.*, 1999; Sekiya-Kawasaki *et al.*, 2002). To further explore roles for *MID2* and *ZEO1* in this pathway we investigated the interactions between *MID2*, *ZEO1* and components of the Rho1p GTPase switch. Sac7p has been demonstrated to be a GTPase activating protein for Rho1p (Schmidt *et al.*, 1997). Strains lacking *SAC7* have a variety of strain-specific phenotypes, and when grown at 18 °C, *sac7Δ* mutants grow slowly and have abnormal actin assembly (Schmidt *et al.*, 1997). A *sac7Δmid2Δ* mutant was constructed and at 22 °C all cells exhibited normal growth. However, at 18 °C, while *sac7Δ* cells had reduced viability, *sac7Δmid2Δ* double mutants grew to wild-type levels (Fig. 6a). Restoration of low-temperature growth in *sac7Δ* cells can also be achieved by deletion of *ROM2* (Schmidt *et al.*, 1997). Conversely,

overexpression of *MID2* in a *sac7Δ* strain leads to inviability (Fig. 6b). In contrast, *ZEO1* appears to play an opposing role to *MID2* in a genetic interaction with Sac7p (Fig. 6c), where a *zeo1* mutant exacerbates the slow growth of a *sac7Δ* mutant at 37 °C. High-copy expression of *ZEO1* does not affect growth of a *sac7Δ* mutant, nor does it suppress the 37 °C growth phenotype (data not shown). Since *SAC7* is required to facilitate the removal of GTP from Rho1p and in effect render the protein inactive, Rho1p is in a constitutively active GTP-bound state in a *sac7Δ* strain, and contributes to cell inviability (Schmidt *et al.*, 1997). Taken together our observations suggest that Mid2p and Zeo1p have opposing actions on Sac7p activity *in vivo*.

The positive regulation of Rho1p is provided by the GDP-GTP exchange factors Rom1p and Rom2p (Ozaki *et al.*, 1996). Although cells lacking *ROM1* do not display obvious phenotypes, *rom2Δ* mutants grow slowly, are prone to lysis at high temperature (Ozaki *et al.*, 1996), and display abnormal mating projection morphology (Manning *et al.*, 1997), suggesting that Rom2p is the major Rho1p GEF in yeast. Since Mid2p and Zeo1p have opposing roles in their effect on *sac7Δ* cells, and since the *mid2rom2* double mutant



**Fig. 6.** *MID2* and *ZEO1* genetically interact with *SAC7*. (a) Serial dilutions of wild-type (WT) BY4742, *mid2Δ*, *sac7Δ* and *mid2Δsac7Δ* mid-exponential-phase cells were spotted onto YPD and incubated at room temperature and 18 °C for 3–5 days. (b) Wild-type (WT) BY4742 cells and *sac7Δ* cells containing either pYES2 vector or pYES2*MID2* were streaked onto selective media containing either glucose or galactose and incubated at 30 °C for 2–3 days. (c) Serial dilutions of wild-type (WT) BY4742, *zeo1Δ*, *sac7Δ* and *zeo1Δsac7Δ* mid-exponential-phase cells were spotted onto YPD medium and incubated at 22 °C or 37 °C for 2–3 days.



**Fig. 7.** *MID2* and *ZEO1* genetically interact with *ROM2*. (a) Tetrad type tetrad from HAB970 (*mid2Δ/MID2 rom2Δ/ROM2*) and HAB974 (*zeo1Δ/ZEO1 rom2Δ/ROM2*) heterozygous diploids grown on YEPD at 30 °C for 2–3 days. (b) Tenfold serial dilutions of wild-type (WT) BY4742 and *rom2Δ* strains carrying vector only (pRS426) or pRS426MID2 grown on YEPD and incubated at the indicated temperatures for 2–3 days.

is synthetically lethal (Table 3), we investigated the interaction of Zeo1p with Rom2p. Results of tetrad analyses generating *zeo1Δrom2Δ* and *mid2Δrom2Δ* double mutants are shown in Fig. 7(a), with the *zeo1Δrom2Δ* mutant largely suppressing the slow growth of a *rom2Δ* single mutant while the *mid2Δrom2Δ* mutant is inviable. These findings are consistent with Mid2p and Zeo1p playing reciprocal roles in signalling to Rho1p. As shown in Fig. 7(b), *MID2* expression from a high-copy vector is able to suppress the temperature-sensitive phenotype of a *rom2Δ* mutant, implying that *MID2* can signal independently of Rom2p.

## DISCUSSION

Our analysis of the synthetic interactions of Mid2p extends recent work (Sekiya-Kawasaki *et al.*, 2002) and indicates that this sensor acts via Rho1p on the *PKC1-MPK1* cell integrity pathway. The synthetic interactions seen with *MID2* and *WSC1*, *ROM2*, *FKS1*, *SMI1* and *FPS1* show that this network upstream of the cell integrity pathway acts to activate 1,3- $\beta$ -glucan synthesis through Rho1p–Fks1p in a way that is Mid2p-independent. Further, the synthetic interactions seen with *MID2* and the 1,6- $\alpha$ -mannosyltransferase encoding *VAN1* and the cell wall mannoprotein encoding *CCW12* suggest that Rho1p may also activate mannoprotein synthesis in a Mid2p-independent fashion. Thus, Mid2p and Wsc1p show distinct signalling patterns, with Wsc1p acting through Rom2p to effect its action (Philip & Levin, 2001). While two-hybrid evidence links

Mid2p and Rom2p, our genetic evidence and that of Sekiya-Kawasaki *et al.* (2002) show that much of Mid2p action is Rom2p-independent. To address the nature of Mid2p signalling we looked for proteins interacting with the cytoplasmic tail of Mid2p; we identified Zeo1p in a two-hybrid screen, and showed that Zeo1p acts to negatively regulate the cell integrity pathway in a Mid2p-dependent fashion.

### Zeo1p is peripherally associated with the plasma membrane

Zeo1p is present in extracts in both soluble and insoluble forms and localizes largely to the cell periphery. Although it lacks a transmembrane domain, Zeo1p localizes to the cell surface, presumably through protein–protein interactions or interactions with the inner surface of the plasma membrane. As Zeo1p localization is not dependent on the presence of either Mid2p or Mtl1p alone, it may bind to both of these plasma membrane proteins, and possibly to others. Null mutants of *ZEO1* resemble *mid2Δ* mutants in being resistant to calcofluor white, with the *mid2Δzeo1Δ* double mutant being no more resistant to calcofluor white than either single mutant, consistent with their operating in a single pathway. The *ZEO1* gene is required for the high-copy *MID2*-induced hypersensitivity to calcofluor white, providing further evidence of a biological link between Zeo1p and Mid2p. Since high-copy expression of *ZEO1* does not induce hypersensitivity to calcofluor white,

it appears that Mid2p requires Zeo1p, but that Zeo1p itself cannot transduce this signal. As the suppression of *MID2*-induced hypersensitivity to calcofluor white by *zeo1Δ* is only partial, there may be other proteins involved in transducing this signal.

Previous work has reported that *zeo1Δ* mutants grow slowly on galactose, and that high-copy *ZEO1* expression leads to Zeocin resistance (MIPS code: HRB113). Zeocin is a copper-chelated glycopeptide antibiotic of the bleomycin family. Although bleomycin antibiotics perturb the plasma membrane of cells, their activity is believed to be primarily due to their ability to bind and degrade DNA. The *ble* gene isolated from the bacterium *Streptoalloteichus hindustanus* encodes a 14 kDa protein that has the ability to bind Zeocin and inhibit its DNA strand cleavage activity (Drocourt *et al.*, 1990; Calmels *et al.*, 1991). Zeo1p has no similarity to the *ble* gene product and it is unclear how *ZEO1* confers resistance to Zeocin in *Saccharomyces cerevisiae*. A number of genomic expression studies show *ZEO1* transcriptional activity to be frequently altered under cell stress (Gasch *et al.*, 2000; Posas *et al.*, 2000). For example, *ZEO1* transcription is increased fourfold in *HOG1*-deleted cells exposed to 0.4 M NaCl for 10 min (Posas *et al.*, 2000). These experiments implicate *ZEO1* in the response of *S. cerevisiae* to cellular stress.

### **Zeo1p affects Mpk1p phosphorylation in a Mid2p-dependent manner**

Phosphorylation of Mpk1p in response to stress is partially dependent on Mid2p (Ketela *et al.*, 1999; Rajavel *et al.*, 1999). Although *zeo1Δ* mutants fail to show a reduction in Mpk1p phosphorylation on heat stress, they do show a constitutive activation of Mpk1p at 22 °C. Thus, Zeo1p may act as a negative regulator for this signalling pathway, with its absence leading to an elevated basal Mpk1p phosphorylation that is Mid2p-dependent. Since the influence of Zeo1p on the calcofluor white phenotype of Mid2p is only partial, other proteins may also modulate these Mid2p responses.

### **Mid2p and Zeo1p signal the cell integrity pathway in a ROM2-independent manner**

In our work several lines of evidence indicate that Mid2p–Zeo1p signalling to the cell integrity pathway can be independent of Rom2p. The pattern of synthetic interactions of *MID2* with *WSC1* and its network with *ROM2* suggests a non-overlapping function for Mid2p, a finding strengthened by the lack of synthetic lethality between *MID2* with *BCK1* or *MPK1*, and indicating that Mid2p acts directly through Rho1p to this cell integrity pathway. In a second line of evidence, the death of *mid2Δ* cells on exposure to mating pheromone appears to be due to insufficient activation of Rho1p, since this phenotype is largely suppressed by over expression of Rho1p (Ketela *et al.*, 1999). Therefore, if activation of Rho1p requires Rom2p, then *rom2Δ* mutants should also be sensitive to mating pheromone. Although *rom2Δ* mutants do exhibit

morphological defects in response to mating pheromone, they remain viable (Manning *et al.*, 1997). Further, over-expression of *ROM2* is unable to suppress the pheromone-induced death phenotype of a *mid2Δ* mutant. Finally, high-copy expression of *MID2* is able to suppress the high-temperature growth phenotype of a *rom2Δ* mutant. Since this phenotype is due to a reduced Rho1p GEF activity, Mid2p can thus signal to Rho1p in a Rom2p-independent manner.

Mid2p and Zeo1p appear to play opposing roles in the regulation of Rho1p. Rom2p has been shown to have a major role in activating Rho1p (Ozaki *et al.*, 1996). Indeed, like *MID2*, *RHO1* is capable of suppressing the temperature sensitivity of a *rom2* mutant. Further, Rom2p (Ozaki *et al.*, 1996) and Mid2p (Phillip & Levin, 2001) stimulate Rho1p-specific GDP–GTP exchange activity. Since the slow growth of *rom2Δ* mutants is at the level of Rho1p, the synthetic lethality of *rom2Δmid2Δ* mutants and the suppression of *rom2Δ* growth defects in a *rom2Δzeo1Δ* double mutant suggest that Zeo1p and Mid2p play reciprocal roles in stimulating Rho1p. The idea that Mid2p and Zeo1p signal at the Rho1p level is further supported by genetic interactions of Mid2p with the Rho1p GTP-activating protein, Sac7p, where *mid2* mutants can rescue the slow growth phenotype of *sac7* mutants at 18 °C and overexpression of *MID2* is lethal in a *sac7Δ* background. Genetic interactions between *MID2*, *ZEO1* and components of the Rho1p GDP–GTP exchange factor and GTP-activating protein suggest that Mid2p acts positively, while Zeo1p acts as a negative regulator of the pathway. For the Mid2p response to calcofluor white, Zeo1p is a critical component of this signalling pathway. Indeed, Phillip & Levin (2001) demonstrated that in response to calcofluor white, GTP loading of Rho1p is increased, suggesting that the Zeo1p control of the Mid2p response to calcofluor white is at the level of the Rho1p GTPase.

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