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Functional characterization of the *Saccharomyces cerevisiae SKN7* and *MID2* genes, and their roles in osmotic stress and cell wall integrity signaling

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Abstract

The yeast *SKN7* gene encodes a transcription factor that is involved in a variety of processes in cell physiology including cell wall synthesis, cell cycle progression, and oxidative stress resistance. Using a transcriptional reporter-based system, it has been demonstrated that Skn7p is regulated by the two-component osmosensor Sln1p in a manner that requires the phosphorelay molecule Ypd1p, but not the response regulator Ssk1p. Consistent with it's regulation by an osmosensor, Skn7p is involved in negative regulation of the osmoresponsive HOG MAP kinase cascade. Cells lacking *SKN7* and the protein serine/threonine phosphatase encoded by *PTC1* are severely disabled for growth, and hyperaccumulate intracellular glycerol. The growth defect of *skn7Δ ptc1Δ* mutants can be bypassed by overexpression of specific phosphatase genes, or by deletion of the HOG MAP kinase pathway-encoding genes *PBS2* or *HOG1*.

MID2 was isolated in a screen designed to identify upstream regulators of Skn7p. Mid2p is an extensively O-mannosylated protein that is localized to the plasma membrane. Mutants with defective β-1,6-glucan synthesis grow more quickly when *MID2* is absent. Conversely, *MID2* is essential for viability in cells lacking *FKS1*, the gene encoding the primary catalytic subunit of β-1,3-glucan synthase. *mid2Δ* mutants are resistant to calcofluor white, a drug that interferes with cell wall chitin synthesis, while

cells overexpressing *MID2* are supersensitive to the drug. *mid2Δ* mutants have a significant reduction in stress-induced chitin synthesis, while cells overexpressing *MID2* hyperaccumulate cell wall chitin. Consistent with a proposed role in sensing and responding to cell wall stress, high copy expression of specific components of the cell wall integrity MAP kinase cascade suppress various *mid2Δ* phenotypes, and Mid2p is essential for full activation of the Mpk1p MAP kinase during various cell wall stress and morphogenic conditions.

Observations from genetic and biochemical experiments suggest that Mid2p is a regulator of the small G-protein encoded by *RHO1*. Deletion of *MID2* is lethal to mutants lacking the Rho1p GEF Rom2p, but suppresses the low temperature growth defect of mutants lacking the Rho1p GAP Sac7p. Conversely, high copy expression of *MID2* is a strong suppressor of mutants lacking *TOR2*, an upstream activator of Rom2p, but is toxic to *sac7Δ* mutants. High copy expression of *MID2* causes increased GEF activity towards Rho1p. Mid2p appears to act in parallel to Rom1p and Rom2p in promoting GDP-GTP exchange for Rho1p in a mechanism that is not yet understood.

Résumé

Le gène *SKN7* code pour un facteur de transcription impliqué dans diverses fonctions de physiologie cellulaire incluant la synthèse de la paroi, la progression dans le cycle cellulaire et la résistance aux attaques d'agents oxydants. L'utilisation d'un système basé sur un rapporteur de transcription a permis de démontrer que Skn7p est régulé par l'osmosenseur à deux composantes Sln1p. Cette régulation requiert la molécule de relais de phosphorylation Ypd1p mais ne nécessite pas la protéine régulatrice de réponse Ssk1p. Conformément avec sa régulation par un osmosenseur, Skn7p est impliqué dans la régulation négative de la cascade de kinases MAP HOG de réponse osmotique. Le gène *PTC1* code pour une phosphatase sérine/thréonine. Les cellules dont les gènes *SKN7* et *PTC1* ont été inactivés sont sévèrement affectées dans leur croissance et accumulent de grande quantité de glycérol intracellulaire. Le défaut de croissance des mutants *skn7Δptc1Δ* peut être surmonté par la surexpression des gènes de certaines phosphatases spécifiques ou par la délétion des gènes *HOG1* ou *PBS2* qui font partie de la cascade de kinases MAP HOG.

MID2 a été isolé lors d'un criblage visant à identifier les régulateurs en amont de Skn7p. Mid2p est une protéine fortement O-mannosilée qui est localisée dans la membrane plasmique. Les mutants affectés dans la synthèse du glucane β-1,6 se

développent plus rapidement lorsque *MID2* est absent. D'autre part, *MID2* est essentiel pour la viabilité des cellules déficientes en *FKS1*, le gène qui code pour la principale sous-unité catalytique de la β-1,3 glucane synthase. Les mutants *mid2Δ* sont résistants au calcofluor, un composé qui interfère avec la synthèse de la chitine de la paroi cellulaire, alors que les cellules qui surexpriment *MID2* sont hypersensibles à ce composé. Les mutants *mid2Δ* ont une réduction importante de la synthèse de chitine induite par le stress alors que les cellules qui surexpriment *MID2* accumulent de grandes quantités de chitine dans leur paroi cellulaire. Conformément avec son rôle proposé dans la détection et la réponse aux stress de la paroi cellulaire, la surexpression des composantes de la cascade de kinases MAP de l'intégrité de la paroi cellulaire supprime divers phénotypes de *mid2Δ*. De plus, Mid2p est essentielle pour une activation complète de la kinase MAP Mpk1p lors de divers stress de la paroi cellulaire et durant la morphogénèse.

Des observations basées sur des expériences génétiques et biochimiques suggèrent que Mid2p est un régulateur de la petite protéine G codée par *RHO1*. L'inactivation de *MID2* est léthale pour les mutants *rom2Δ*, qui code pour le GEF de Rho1p, mais supprime le défaut de croissance à basse température des mutants *sac7Δ*, qui code pour le GAP de Rho1p. D'autre part, l'expression de multiples copies de *MID2* est un suppresseur efficace des mutants *tor2Δ*, qui code pour un activateur en amont de Rom2p, mais est toxique pour les mutants *sac7Δ*. L'expression de multiple copies de

MID2 cause une augmentation de l'activité GEF envers Rho1p. Mid2p semble agir en parallèle de Rom1p et de Rom2p en favorisant l'échange GDP-GTP chez Rho1p grâce à un mécanisme encore inconnu.

Traduction par Nicolas Pagé

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Bussey boys Jeff Brown, Bo Jiang and Terry Roemer who were an inspiration during my first months and years in the lab.

Finally, I'd like to thank my parents, who have been unfailingly supportive in so many ways.

Preface

This thesis has been assembled in accordance with the guidelines of the Faculty of Graduate Studies and Research of McGill University. It consists of an abstract and résumé, an introduction and literature review (Chapter 1), modified versions of two previously published manuscripts (Chapters 2, 3) a section consisting of data that will be assembled into a manuscript for publication (Chapter 4), and a brief summary and discussion of the data contained in the thesis (Chapter 5).

Here I present a contribution to original knowledge that includes: the identification of one aspect of the upstream regulatory apparatus for the Skn7p transcription factor; the characterization of and identification of function for Mid2p; and the identification of Mid2p as a regulator of the small GTP-binding protein Rho1p. The results contained within this thesis represent a significant advance in the study of eukaryotic signal transduction in two distinct stress response pathways.

The design of experiments and the results represented here are the result of my own effort with the following exceptions: Bo Jiang first identified the *ptc1Δ skn7Δ* phenotype; R. Green first identified WSC1 as a high copy suppressor of fks1Δ mid2Δ mutants; all DNA sequencing was performed by S. Vérroneau and P. Ménard; and R. Stewart and J. Brown constructed the D427N and D427E alleles of Skn7p and constructed the LexA-Skn7 fusion.

Chapter 2 has been published as a paper by T. Ketela, J.L. Brown, R.C. Stewart and H. Bussey (1998) in Molecular and General Genetics 259:372-378. Chapter 3 has been published as a paper by T. Ketela, R. Green, and H. Bussey (1999) in the Journal of Bacteriology 181:3330-3340.

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List of Abbreviations

3AT	3-Amino-1,2,4-Triazole
5-FOA	5-fluoroorotic acid
ATP	adenosine triphosphate
bp	base pair
cAMP	adenosine 3',5'-cyclic phosphoric acid
DAG	diacylglycerol
DH	Dbl-homologous
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ER	endoplasmic reticulum
GAP	GTPase activating protein
GDP	guanosine 5'-diphosphate
GEF	GDP-GTP exchange factor
GFP	green fluorescent protein
GTP	guanosine 5'-triphosphate
HA	hemagglutinin

HOG	high osmolarity glycerol
HRP	horse radish peroxidase
IP_3	inositol-1,4,5-triphosphate
kb	kilobase
kD	kilo Dalton
KOH	potassium hydroxide
LSS	low speed spin
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MES	4-morpholineethanesulfonic acid
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
O-linked	serine/threonine linked
ORF	open reading frame
PCR	polymerase chain reaction
PH	pleckstrin homology
PI	phosphotidylinositol
PMSF	phenylmethylsulphonylfluoride

RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STRE	stress response element
UAS	upstream acting sequence
YEPD	yeast extract, peptone, dextrose
YNB	yeast nitrogen base

CHAPTER 1

Introduction to the High Osmolarity Response Pathway, and the Extracellular Matrix Synthesis and Regulation Machinery of *Saccharomyces cerevisiae*

The High Osmolarity Response Pathway of *Saccharomyces cerevisiae*

1.1 High Osmolarity Triggers a MAP Kinase Cascade

Maintaining a positive osmotic gradient across the plasma membrane is a vital requirement for sustaining cell viability in all organisms. Unless cells employ measures to counteract changed external osmolarity conditions, they can experience swelling and even cell lysis due to immersion in a hypotonic environment, or severe cell shrinkage due to dehydration when their external environment is hypertonic. Unicellular organisms are directly exposed to the environment, and thus must be able to swiftly and efficiently cope with rapid fluctuations in osmolarity in their immediate surroundings. The budding yeast *Saccharomyces cerevisiae* frequently colonizes the surfaces of fruits (Mortimer *et al.* 1994). As fruits ripen and dry and sugars become concentrated, this environment becomes hypertonic. Collection of water through precipitation on the fruit surface can rapidly dilute osmolytes, shifting the yeast's surrounding to a hypotonic state.

Augmentation of the intracellular glycerol pool plays a crucial role in enabling cellular adaptation to increased external osmolarity in *S. cerevisiae* (Brown 1978). Mutant cells that are unable to accumulate sufficient glycerol in response to osmotic stress become inviable when cultured in high osmolarity conditions (Albertyn *et al.* 1994). Cytosolic accumulation of glycerol increases intracellular turgor pressure, and restores the osmotic gradient between the intra and extracellular environments. *GPD1* encodes a protein with NADH-dependent glycerol-3-phosphate dehydrogenase activity, and Gpd1p is the principal source of intracellular glycerol in *S. cerevisiae*. Increased accumulation of *GPD1* mRNA and Gpd1 protein occurs in response to osmotic shock. The induction of *GPD1* transcription is mediated by a MAP (Mitogen Activated Protein) kinase cascade that is responsive to osmotic stress (Albertyn *et al.* 1994), the components of which are described below.

The osmo-responsive signal transduction pathway contains four protein kinases whose hierarchical position within the cascade have been defined by both genetic and biochemical approaches. The distal tier of the pathway contains two homologous MAPKK kinases, Ssk2p and Ssk22p. These two proteins have overlapping activity in activating the MAPK kinase Pbs2p (Maeda *et al.* 1995). In turn, Pbs2p is responsible for activating the MAP kinase Hog1p (Brewster *et al.* 1993). While Ssk2p and Ssk22p possess only serine/threonine protein kinase activity, Pbs2p is responsible for phosphorylation of both specific threonine (Thr174) and tyrosine (Tyr176) amino acid residues within Hog1p.

The appearance of tyrosine phosphorylation on Hog1p is rapid, occurring in one minute or less after exposure of cells to high external osmolarity. Phosphorylation of Hog1p peaks roughly 10-15 minutes after initiation, and then declines over the next 10-30 minutes (Brewster *et al.* 1993, Maeda *et al.* 1995). After a longer period, even in the continuous presence of high external osmolarity conditions, discernible phosphorylation of Hog1p is not present, suggesting that cells have accomplished the task of osmoadaptation, and pathway signaling is diminished.

Although the extent of tyrosine phosphorylation on the cellular pool of Hog1p is commonly used as an indicator of Hog1p kinase activity, activation of Hog1p apparently requires phosphorylation of both Thr174 and Tyr176 since mutations that cause constitutive tyrosine phosphorylation of Hog1p do not result in constitutive Hog1p activity (Jacoby *et al.* 1997, Wurgler-Murphy *et al.* 1997). Although cells that have been deleted for either *PBS2* or *HOG1* have no detectable growth defects when grown on medium of low osmolarity, when shifted to medium of high osmolarity, both mutants display morphological abnormalities including enlarged cells and elongated buds. If osmolarity of the growth medium is sufficiently high (i.e.>0.3M NaCl), *hog1Δ* and *pbs2Δ* mutants become inviable (Brester *et al.* 1993).

1.2 Downstream Effectors of the HOG Pathway

The inability of cells that have been mutationally disrupted for Hog1p activation to survive high osmolarity stress can be directly related to insufficient transcriptional induction of genes whose products are required to combat osmostress. In addition to *GPD1*, several other genes are transcriptionally up-regulated during osmostress including *CTT1* (important for detoxification of super oxide radicals) (Marchler *et al.* 1993, Schüller *et al.* 1994), *ENA1* (required for ATP-dependent Na+ efflux pumping) (Wieland *et al.* 1995, Marquez and Serrano 1996), *HSP12* (appears to be required for switching from carbohydrate to fatty acid utilization) (Varela *et al.* 1995), and *GAC1* (regulates Glc7p phosphatase activity) (Parrou *et al.* 1997). In addition to modifying the intracellular environment so that they can cope with osmotic stress, cells induce changes in the cell wall, perhaps to accommodate the alteration of cell volume that results from exposure to hypertonic environments. Jiang *et al.* (1995) demonstrated that transcription of *EXG1*, a β-exoglucanase is partially under the control of Hog1p.

Many of the genes that are transcriptionally up-regulated by stress, such as *CTT1* and *HSP12*, contain an STRE (Stress REsponsive Element) upstream acting element (UAS) in their promoter. Activity in the HOG pathway stimulates the transcription of some genes like *HSP12* and *CTT1* via the STRE UAS (Schüller *et al.* 1994, Varela *et al.* 1995). Activity in the cAMP-dependent kinase pathway antagonizes stress responsive gene transcription via the same upstream promoter element (Marchler *et al.* 1993, Smith *et al.* 1998). The homologous Msn2p and Msn4p zinc finger transcription factors are known to control the transcription of several genes via the STRE UAS, and mutants lacking *MSN2* and *MSN4* are sensitive to a variety of conditions including carbon source starvation, heat shock, and osmotic and oxidative stress (Martínez-Pastor *et al.* 1996). A direct link between between Hog1 and Msn2p/Msn4p is not yet established, but it appears that Hog1p may at

least partially regulate osmotic stress-induced gene transcription through Msn2p and Msn4p.

Hog1p also regulates the activity of two transcriptional repressors, Sko1p and the Ssn6-Tup1 complex. Sko1p mediates Hog1p-dependent transcription of *ENA1* by releasing from the cAMP response element (CRE) of the *ENA1* promoter during osmostress (Proft and Serrano 1999). In unstressed conditions, the Ssn6-Tup1 complex represses the transcription of several osmotic stress-induced genes including *HAL1*, *GPD1*, *CTT1*, *ALD2*, *ENA1* and *SIP18*. During periods of osmotic stress, in a Hog1p-dependent manner, Ssn6-Tup1 releases from the CRE, and transcriptional repression of these genes is relieved (Márquez *et al.* 1998).

In order to phosphorylate its transcription factor targets, Hog1p must enter the nucleus. Hog1p entry into the nucleus is limited to when it is in a phosphorylated state (Ferrigno *et al.* 1998, Reiser *et al.* 1999). Hog1 nuclear import requires the small GTP-binding protein Gsp1p and the importin homolog *NMD5*, and its export from the nucleus requires the activity of the nuclear export signal receptor Xpo1p (Ferrigno *et al.* 1998). The Hog1p activators Pbs2p and Ste11p do not shuttle to the nucleus, so presumably, activation of Hog1p by Pbs2p takes place solely in the cytoplasm (Ferrigno *et al.* 1998).

1.3 The Upstream Regulatory Machinery of the HOG Pathway

In bacteria, two-component systems govern widely diverse processes such as locomotor response to chemotactic signals and fruiting body formation. In the two-component paradigm, signal transduction is accomplished by two units; a sensor/receptor molecule and a response-regulator protein (Parkinson and Kofoid 1992). Most sensor modules are anchored within the cell membrane via two transmembrane domains. Separating the membrane-spanning regions, and exposed to the extracellular environment, is a domain that appears to act as a stimulus sensing module. Although some sensor domains

appear to bind specific ligands, the precise stimuli that most sensor regions respond to remain to be identified. When activated by a stimulus, a specific histidine residue in an intracellular kinase domain becomes phosphorylated. The phosphate molecule is then relayed to a specific aspartic acid residue in the soluble response regulator protein. While unphosphorylated, response regulator proteins are typically in an inactive state. Upon phosphorylation, the response-regulator becomes operative, and is able to perform its designated function. Response regulator proteins are often, but not exclusively, regulators of transcriptional output. Those that do regulate transcription typically contain domains that mediate DNA binding and transcriptional activation/repression. The two-component theme is utilized extensively by bacteria, with some species having 50 or more separate two-component systems operating within a single cell. Until very recently, it appeared that two-component systems were exclusive to bacteria. However, it is now known that a diverse array of eukaryotic species including fungi, and plants contain two-component signaling modules (Chang *et al.* 1993, Shieh *et al.* 1997).

S. cerevisiae SLN1 was among the first genes reported to encode a eukaryotic homolog of bacterial two-component proteins (Ota and Varshavsky 1993). *SLN1* encodes a predicted plasma membrane localized osmosensor that governs activity in the HOG MAP kinase cascade (Maeda *et al.* 1994). The N-terminal third of Sln1p contains two transmembrane domains, linked by a region exposed to the extracellular environment. The C-terminal half of Sln1p contains two domains, one of which exhibits strong sequence similarity to bacterial two-component histidine kinase motifs, and the other which is similar to bacterial two-component response regulator domains. The histidine kinase domain spans residues 546-912 of Sln1p, and the response-regulator region is situated between residues 1085 and 1220. Recent evidence suggests that Sln1p may not directly sense increased external osmolarity, but instead perceives imbalance between intracellular and extracellular osmotic pressure (Tao *et al* 1999).

Genetic experiments and in vitro studies have demonstrated intermolecular phosphoryltransfer between Sln1p molecules (Maeda *et al.* 1994, Posas *et al.* 1996). Phosphates are reciprocally relayed from H576 to D1144 within a pair of SLN1 proteins. Phosphate molecules are then relayed to another histidine residue (H64) within the histidine kinase domain of a separate, small, soluble protein, Ypd1p (Posas *et al.* 1996). Ypd1 appears to act as a shuttle for phosphate molecules, receiving them from Sln1p, and delivering them to a specific aspartic acid residue (D554) within a third two-component module, Ssk1p (Posas *et al.* 1996).

Curiously, in contrast to the paradigm for bacterial response regulator proteins, phosphorylation renders Ssk1p inactive. In fact, phosphate molecule flow through the whole yeast two-component pathway is regulated differently than most eukaryotic signaling pathways. During low or no osmostress periods, Sln1p kinase activity is high, and consequently, Ssk1p is maintained in a phosphorylated, inactive state. Sln1p kinase activity ceases when cells are exposed to high external osmolarity, permitting the accumulation of unphosphorylated, active Ssk1p. In its operative state, Ssk1p interacts with the N-terminal auto inhibitory regions of Ssk2p and Ssk22p (Posas and Saito 1998). This interaction permits Ssk2p and Ssk22p to become active as protein kinases, thereby initiating signaling within the HOG MAP kinase cascade.

Activation of Ssk1p resulting from cessation of Sln1p kinase activity occurs rapidly (<1 min.), suggesting that phosphate molecules are quickly lost from Ssk1p during osmostress. In bacteria, half lives for response regulator phosphorylation range from seconds for CheY and CheB (Hess *et al.* 1988, Wylie *et al.* 1988) to ~10-12 hours for VanR (Wright *et al.* 1993). Recently, Janiak-Spens *et al.* (1999) have demonstrated that the half-life of Ssk1p phosphorylation in vitro is ~42 hours, by far the longest yet observed for any two-component response regulator protein. Since Ssk1p is able to initiate signaling within the HOG pathway in a much shorter (~2500 fold) time period than the half life

duration of its phosphorylation, it is possible that a specific aspartyl phosphatase activates Ssk1p. However, a molecule with this activity has not yet been identified in *S. cerevisiae*.

Mutations that block phosphorylation of Ssk1p, such mutation of D554, or deletion of Sln1p or Ypd1p, result in constitutive Ssk1p activity (Maeda *et al.* 1994, Posas *et al.* 1996). The resulting constitutive signaling within the HOG MAP kinase cascade, which occurs even in low osmolarity medium, is highly deleterious to cells, and results in severe growth defects or even inviability. Hyperactivity of Ssk1p can be blocked by deleting downstream positive acting elements of the pathway such as *HOG1*, *PBS2*, or *SSK2* and *SSK22*, or by over-expressing negative regulators of pathway signaling such as protein phosphatases (see section titled Negative Regulation of HOG Pathway Signaling).

In the orthodox MAP kinase pathway paradigm, cascade signaling is initiated by stimulation of the MAPKK kinase tier, and the signal is then propagated down through the MAPK kinase to the MAP kinase. The model presented thus far suggests that the HOG pathway conforms to this model. However, the presence of a second osmosensor that activates Hog1p independently of the MAPKK kinase tier was inferred from the observation that mutants lacking both Ssk2p and Ssk22p could still sense and respond to osmostress. *SHO1* (Synthetic High Osmolarity-sensitive), which encodes a second osmosensor, was isolated in a screen for mutants that eliminated the ability of *ssk2Δ ssk22Δ* mutants to respond to osmostress (Maeda *et al.* 1995). Sho1p, has four predicted membrane spanning domains and a C-terminal SH3 domain, and contains no regions of homology to two-component proteins. The SH3 domain of Sho1p binds to an N-terminally located proline-rich SH3-binding motif in Pbs2p. However, while the interaction between Pbs2p and Sho1p is necessary, it is not sufficient for Pbs2p activation. Two additional proteins, Ste11p and Ste50p participate in the Sho1p-dependent activation of Pbs2p. Ste11p a MAPKK kinase component of the mating pheromone response pathway is responsible for Sho1p-dependent phosphorylation of Pbs2p (Posas and Saito 1997. Like the Ssk2p and Ssk22p MAPK kinases, Ste11p contains an N-terminal auto-inhibitory

domain. Ste50p's role during osmostress is to physically interact with Ste11p, preventing the auto inhibitory region from interfering with Ste11p protein kinase activity (Posas *et al.* 1998).

The Sho1p sensor appears to require higher external osmolarity conditions than Sln1p does for activation, and it is slower to effect activation of Hog1p than Sln1p (Maeda *et al.* 1995). The benefit of employing two distinctly dissimilar sensors that have different sensitivities and response times may be that such a system is sensitive to a wide range of osmotic imbalances, and is able to provide more precise regulation of Hog1p activity according to the severity of osmolarity imbalance presented to the cell than could be accomplished by a single sensor. An arrangement such as this may be of particular benefit to organisms like *S. cerevisiae* which inhabit environments prone to rapid and large fluctuations in osmolarity.

1.4 Negative Regulation of HOG Pathway Signaling

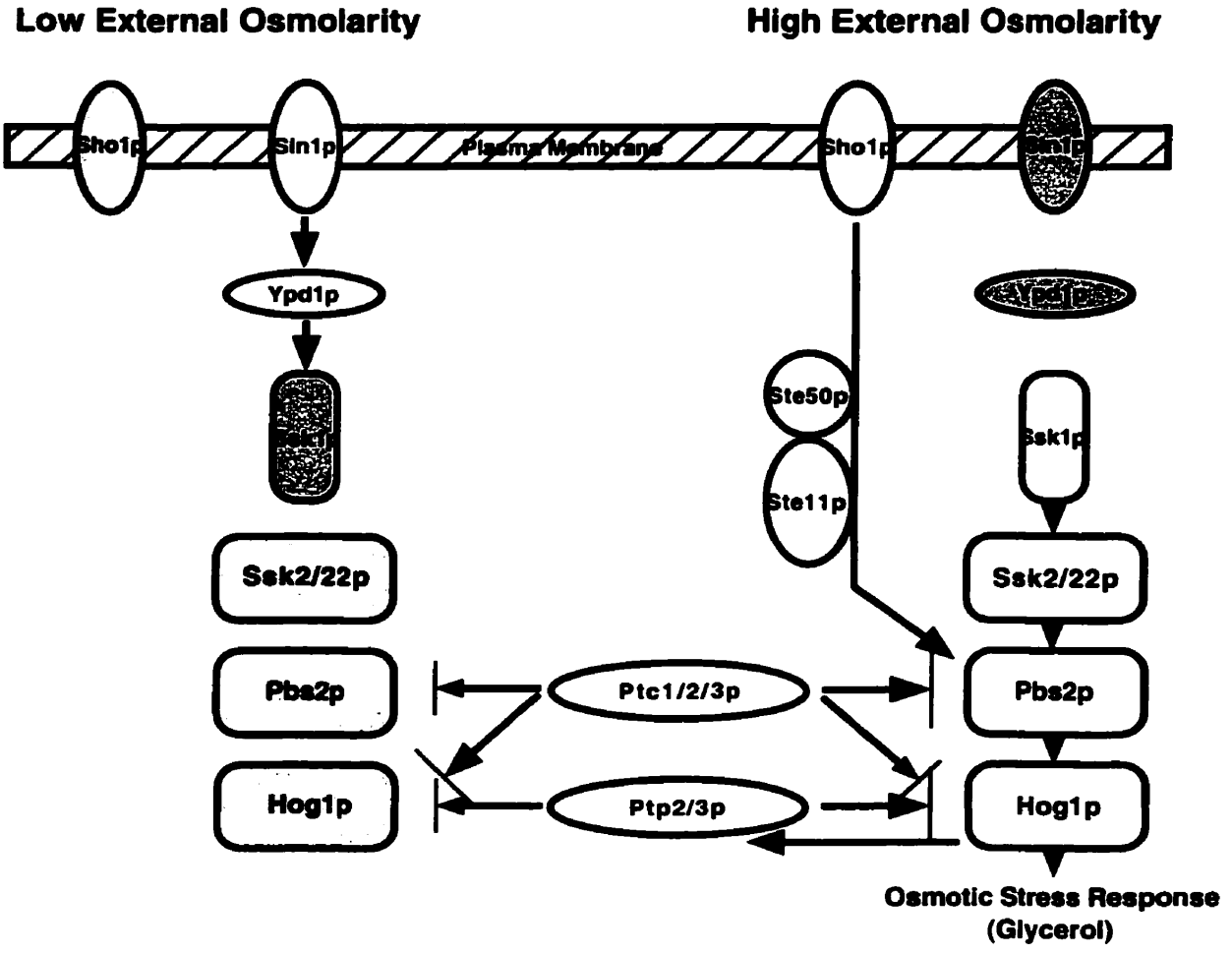
Since persistence of Hog1p activity in the absence of osmotic stress is deleterious to cell growth, it would be expected that pathway signaling occurs only as long as is necessary to counteract osmotic imbalance. To ensure rapid cessation of HOG pathway activity after signals originating from the two osmosensors terminate, *S. cerevisiae* employs protein phosphatases to dephosphorylate, and thus deactivate MAP kinase pathway components. The serine/threonine phosphatase Ptc1p and the homologous Ptc3p have been implicated in dephosphorylation of Pbs2p. Ptc1p possibly also dephosphorylates Hog1p, probably at the site of Pbs2p-dependent phosphorylation (Thr 174) (Maeda *et al.* 1993, Maeda *et al.* 1994). Removal of tyrosine phosphorylation on Hog1p (Tyr 176) is accomplished by at least two phosphatases; Ptp2p and the homologous Ptp3p (Wurgler-Murphy *et al.* 1997, Jacoby *et al.* 1997).

Single deletions of Pbs2p or Hog1p phosphatase-encoding genes do not result in substantial growth defects, presumably because the remaining phosphatases are able to compensate. However, simultaneous loss of two phosphatases, for example *PTC1* and *PTP2*, results in severe growth defects or even cell inviability due to constitutive, endogenous Hog1p activity. The growth defects in *ptc1Δ ptp2Δ* cells can be almost entirely bypassed by deleting either *HOG1* or *PBS2* (Ketela *et al.* 1998).

Additional complexity was added to the understanding of pathway regulation when it was discovered that transcription of *PTP2* and *PTP3* is up-regulated during periods of osmostress (Wurgler-Murphy *et al.* 1997, Jacoby *et al.* 1997). Induction of *PTP2/3* transcription is directly dependent on Hog1p, suggesting the existence of a feedback loop. During the osmostress response, phosphorylated Hog1p acts to up-regulate *PTP2* and *PTP3*, the gene products of which in turn, act to down-regulate Hog1p. While an intra/extracellular osmotic imbalance exists, positive signal to Hog1p is sufficiently strong to overcome phosphatase activity. Once the osmotic imbalance is rectified, positive signal to Hog1p from the osmosensor system is reduced and is no longer able to overcome the negative effect of the phosphatase pool that has accumulated during the period of Hog1 activity, and Hog1p kinase activity is rapidly squelched. Together, the multiple mechanisms of positive and negative regulation that govern Hog1p activity result in a signal transduction pathway that is exquisitely sensitive and responsive.

Figure 1.1 Model of the HOG Pathway: Signaling and Regulation. Shaded objects represent pathway elements that are in an inactive state. Unshaded objects represent the active forms of proteins.

The High Osmolarity Glycerol (HOG) Pathway



1.5 *SKN7* Encodes a Fourth *S. cerevisiae* "Two-Component" Protein

KRE9 encodes a small, secreted peptide of unknown biological activity. Cells lacking Kre9p grow extremely slowly, have morphological defects and have a substantial reduction in cell wall alkali-insoluble ß-1,6-glucan content (Brown *et al.* 1993). In an attempt to learn more about the function of Kre9p, a screen for high copy suppressors of *kre9Δ* mutants was initiated. Suppressor of *Kre* Null number 7 (*SKN7*) was isolated in this screen and characterized (Brown *et al.* 1993). *SKN7* encodes a transcription factor with a number of notable features, including an N-terminal DNA-binding domain, a central coiled-coil domain, an adjacent region with strong homology to two-component response regulator domains, and a glutamic acid-rich C-terminal tail.

The observation that suppression of *kre9Δ* mutants by *SKN7* depended on an intact response regulator domain, and that specific mutations at the putative phosphoryl receiver site (D427) affected Skn7p-dependent transcription of reporter genes, led Brown *et al.* (1994) to suggest that the response regulator domain of Skn7p is phosphorylated by a histidine kinase, and this modification is functionally important to Skn7p. It is not yet known how high copy expression of *SKN7* bypasses the *kre9Δ* mutant phenotype. Functional Skn7p is essential in mutants lacking *PKC1* (see page 19), and high copy *SKN7* is able to suppress the lysis defect of *pkc1Δ* mutants (Brown *et al.* 1994), suggesting that Skn7p affects transcription of cell wall synthesis genes. Additionally, there is some evidence that Skn7p participates in regulation of chitin synthesis and cell wall mannoprotein synthesis (S. Nagahashi, J. Horeka, personal communication).

In subsequent work, Skn7p has been demonstrated to participate in a number of different physiological processes. Morgan *et al.* (1995) isolated *SKN7* as a dosage suppressor of mutants lacking either SBF or MBF, the central regulators of G1 cyclin transcription. In SBF and MBF mutants, high copy *SKN7* is able to restore G1 cyclin expression through an unknown mechanism. In addition, *skn7Δ* mutants are hypersensitive

to certain oxidative stresses, such as exposure to hydrogen peroxide (Krems *et al.* 1996). Skn7p is required for the transcriptional activation of some genes required to combat oxidative stress, including *TRX2*, *TRR1* (Morgan *et al.* 1997). For several oxidative stress-induced proteins, Skn7p acts in cooperation with Yap1p, a bZIP transcription factor of the AP-1 family, to regulate their transcription (Lee *et al.* 1999). Interestingly, mutational disruption of the two-component response regulator domain in Skn7p does not affect its ability to modulate the oxidative stress response (Morgan *et al.* 1997). This finding suggests Skn7p activity is regulated by multiple mechanisms, and that Skn7p's role in oxidative stress is modulated independently of histidine kinase activity.

Although Skn7p is known to directly bind DNA, and promoter regions that are responsive to Skn7p have been identified (Lee *et al.* 1999), the exact sequence that Skn7p recognizes has not yet been ascertained. Finally, Skn7p interacts with the small, GTP-binding protein Rho1p (Alberts *et al.* 1998). The significance of this interaction is not yet clear, but it is possible that it may be related to Skn7p's putative role in regulation of cell wall synthesis genes (see section titled Upstream Regulators Of The Cell Integrity Pathway for information on Rho1p and its role in cell wall synthesis).

The Cell Wall and Cell Wall Integrity Pathway of *Saccharomyces cerevisiae*

1.6 Biosynthesis of the Cell Wall

The yeast cell wall is an essential organelle that comprises roughly 30% of cell dry weight. This structure performs a number of functions, including; maintaining cellular shape, acting as a barrier against external threats to cell integrity, serving as a scaffold for enzymes important to cell nutrition and growth, and protection of osmotic integrity.

The cell wall is primarily comprised of four components: β-1,3-glucan, β-1,6-glucan, mannoprotein, and chitin. β-glucan (proportionally ~80% β-1,3-linked and ~20% β-1,6-linked) and mannoprotein each make up roughly half of the wall weight, while chitin comprises only a small fraction (~2-3%) (Klis 1994).

β-1,3-glucan plays an essential role by providing structural rigidity to the wall. β-1,3-glucan chains, typically ~1500 glucose residues long, largely consists of a linear chain of β-1,3-linked glucose residues with a small number (~3%) of β-1,6-linked residues residing at branch points (Manners *et al.* 1973). Synthesis of β-1,3-glucan is carried out by a plasma membrane-bound multimeric enzyme complex (Douglas *et al.* 1994, Eng *et al.* 1994, Ram *et al.* 1995). When glucose is the primary carbon source, the principal catalytic component of the synthase complex is the product of the *FKS1* gene, Fks1p. Fks1p-mediated β-1,3-glucan synthase activity is dependent on physical interaction of Fks1p with the small RHO-type GTPase Rho1p (Qadota *et al.* 1996, Drgonova *et al.* 1996) (see subsequent sections for more information about Rho1p). Expression of *FKS2*, the product of which has 87% sequence identity to Fks1p, is limited to certain stress conditions, carbon sources other than glucose, and sporulation (Mazur *et al.* 1995). On glucose, cells lacking *FKS1* have a substantial reduction in cell wall β-1,3-glucan content, display abnormal morphology, and have a slow growth phenotype while cells disrupted for *FKS2* have no

obvious phenotype. Transcription of *FKS2* is up-regulated in mutants lacking *FKS1*, and increased production of Fks2p presumably accounts for the remaining β-1,3-glucan content and viability of *fks1Δ* mutants. Cells lacking both *FKS1* and *FKS2* are inviable under any circumstances.

Although it is considerably less abundant than β-1,3-glucan, β-1,6-glucan is essential for cell wall integrity. It is thought to act as a "glue-like" molecule in the wall, cross-linking with all of the other polysaccharide polymers (Kollar *et al.* 1997). β-1,6-glucan molecules, averaging 140-200 glucose residues in length, are highly branched, and consist predominately of β-1,6-linked glucose, along with a small proportion of 1,3-linked residues (Boone *et al.* 1990). Genetic screens for mutants resistant to the K1 killer toxin, which binds to a cell wall receptor that contains β-1,6-glucan, have yielded several *KRE* (killer resistant) genes, many of which are required for β-1,6-glucan synthesis. Although a model has been proposed in which β-1,6-glucan is produced by a stepwise process in the secretory pathway, beginning with chain initiation in the ER by Kre5p, chain elaboration by Kre6p/Skn1p in the golgi, and Kre1p and Kre9p-dependent chain maturation at the cell surface, the exact process by which β-1,6-glucan is synthesized is not yet known.

Chitin is a linear polymer composed of β-1,4-linked N-acetylglucosamine residues. Chitin synthesis in *S. cerevisiae* is accomplished by three different enzymes; Chs1p, Chs2p, and Chs3p. Chs1p appears to function as a repair enzyme, and is required to compensate for chitin lost to chitinase (Cts1p) digestion of the cell wall during cell separation (Cabib *et al.* 1992). Although Chs1p provides the major chitin synthase activity in vitro, *chs1Δ* mutants contain wild type levels of chitin in vivo, and grow well in rich medium (Bulawa *et al.* 1986). In acidic medium, *chs1Δ* cells have a slight lysis phenotype, presumably due to low pH-dependent activation of chitinase. Chs2p is responsible for synthesis of the primary cell septum (Silverman *et al.* 1988). Cells lacking Chs2p show a slight reduction in chitin content, but have severe growth and morphological defects. Chs3p is responsible for the bulk (90-95%) of cellular chitin (Bulawa 1992). Most chitin synthesized by Chs3p is

deposited at birth and bud scars, while a smaller amount is spread throughout the lateral wall. Mutants lacking *CHS3* have very low levels of cell wall chitin (~10% of normal), but are able to grow well. Chs3p also plays a role in spore formation. Chitin produced by Chs3p is deactylated to form chitosan (Mishra *at el.* 1997), which is deposited in the spore coat (Pammer *et al.* 1992). Regulation of Chs1p and Chs2p activity appears to involve both transcriptional and posttranslational controls (Pammer *et al.* 1992, Choi *et al.* .1994, Uchida *et al.* 1996). Although transcription of *CHS3* varies with the cell cycle (Pammer *et al.* 1992), it does not contribute to regulation of Chs3p activity (Choi *et al.* 1994, Chuang and Schekman 1996, Cos *et al.* 1998). Instead Chs3p appears to be regulated by its association with accessory proteins.

Localization and activation of Chs3p requires several proteins. Trilla *et al.* (1997) determined that the gene product of *CHS4* is required for post-translational activation of Chs3p. Chs4p is also required for the localization of Chs3p to the mother-bud neck. In this process, Chs4p appears to link Chs3p to the septin ring by binding to the septin-associated protein Bni4p (DeMarini *et al.* 1997). Chs5p is required for the polarized transport of vesicles that carry, among other proteins, Chs3p, towards the plasma membrane (Santos and Snyder 1997). In a subsequent step, Chs6p is necessary for the anterograde transport of Chs3p from vesicles to the plasma membrane (Ziman *et al.* 1998). Chs7p is required for the transport of Chs3p from the ER (Trilla *et al.* 1999). *chs7Δ* mutants retain Chs3p in the ER, resulting in reduced Chs3p-dependent chitin synthesis. The inability to elevate chitin synthesis in vivo by overexpressing CHS3 appears to be caused by cell's inability of cells to export excess Chs3p from the ER. The export of Chs3p from the ER appears to be limited by Chs7p since co-overexpression of *CHS7* and *CHS3* causes increased chitin synthesis in vivo.

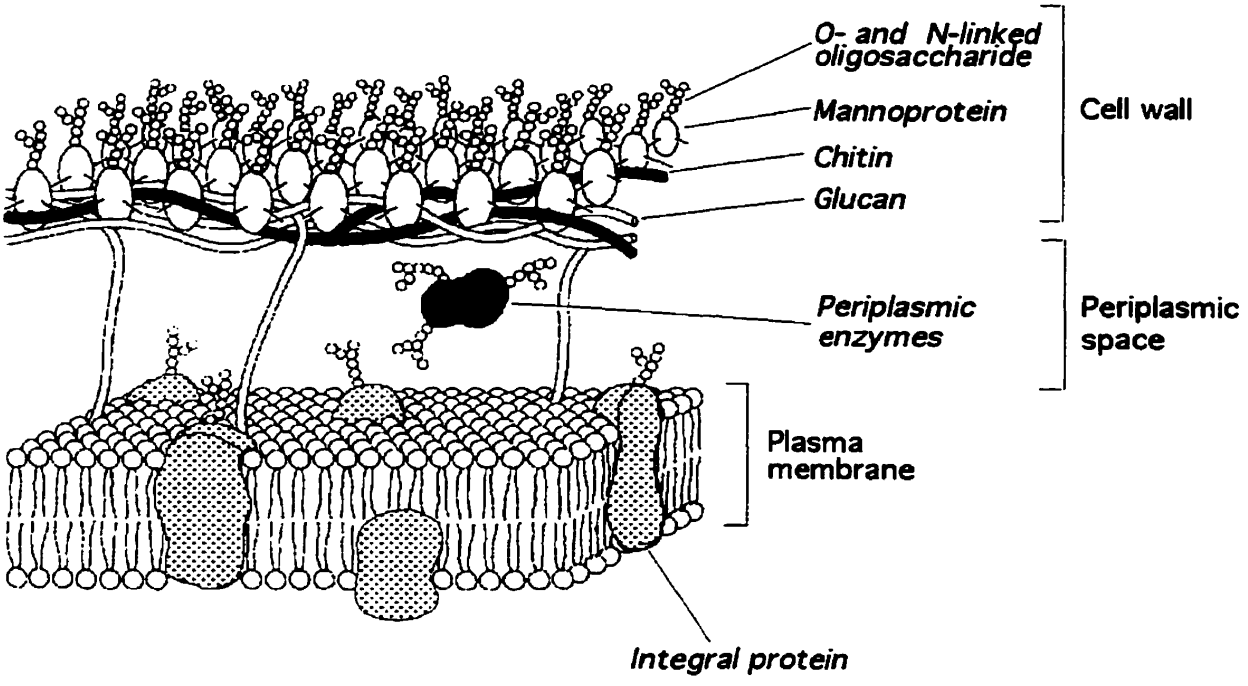
A large proportion of glycosylated proteins in *S. cerevisiae* reside in the cell wall. These molecules, often collectively refered to as mannoproteins, are modified, sometimes extensively, by the post-translational addition of different carbohydate chains. Since this

thesis is not directly related to protein mannosylation, I will deal only briefly with this subject. O-linked mannosylation refers to the addition of two to five mannose residues onto serine and threonine amino acid residues of a secreted protein, or the extracellular portion of a plasma membrane bound protein. Addition of the first mannose by members of the PMT family occurs in the ER (Gentzsch and Tanner 1996, 1997), while subsequent addition of mannose, the first two of which are α1,2 linkages, and the second two of which are α1,3-linkages, takes place in the golgi apparatus by members of the KRE2 family, and Mnn1p (Haüsler *et al.* 1992, Lussier *et al.* 1997).

N-linked glycosylation is initiated in the ER where a lipid-linked oligosaccharide is transferred to selected asparagine residues of specific proteins that are travelling through the ER. Three glucose resides are removed from the inner chain by glucosidases I and II before the protein exists the ER and enters the golgi apparatus. In the golgi apparatus, the N-linked core may then be extended by the addition of up to 200 mannose residues. The backbone of the outerchain consists of roughly 50 α1,6-linked mannose units (Hersovics and Orlean 1993). This structure may become highly branched by the addition of α1,2 and α1,3-linked mannoses.

Figure 1.2 A schematic model of the *S. cerevisiae* extracellular matrix. Adapted by M. Lussier from Watson *et al.*, Molecular Biology of the Gene, fourth edition.

The Yeast Cell Surface



1.7 Cell Wall Integrity is Modulated By a MAP Kinase Cascade

The cell wall is a dynamic structure. Cell wall construction must be coordinated with such processes as the vegetative budding cycle, mating projection formation, ascospore generation, and wall damage reparation. Significant understanding of the mechanism that regulates cell wall synthesis and maintains cell wall integrity was gained with the discovery that cells containing mutations in *PKC1*, a gene encoding the budding yeast homolog of mammalian Protein Kinase C, display a cell lysis phenotype consistent with defects in cell wall construction (Levin and Bartlett-Heubusch 1992, Levin *et al.* 1994).

Pkc1p is a 1151 amino acid residue serine/threonine protein kinase. Like mammalian isoforms of PKC, yeast Pkc1p contains a pseudosubstrate site, and putative domains for binding diacylglycerol, Ca^{2+} and phospholipids. In mammalian cells, extracellular signals result in diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) production. DAG acts as a second messenger to activate PKC, and IP_3 causes the release of Ca2+ from intracellular stores. The pseudosubstrate region is thought to keep PKC proteins in an inoperative state in the absence of an activating signal (Hug and Sarre, 1993).

The role of cofactors in activation of yeast Pkc1p is not well understood. Although Antonsson *et al.* (1994) reported that phospholipid or diacylglycerol do not activate yeast Pkc1p, Stt4p, a phosphatidyl 4-kinase, has been demonstrated to function upstream of Pkc1p (Yoshida *et al.* 1994). Additionally, the presence of phosphatidyl serine is necessary for the activation of Pkc1p by the Rho1p small GTPase in vitro (Kamada *et al.* 1996).

Cells lacking functional Pkc1p can only grow in the presence of an osmotic stabilizer, are sensitive to caffeine, staurosporine, and high temperature, and experience hyperrecombination of DNA. When shifted to hypoosmotic medium, *pkc1Δ* mutants arrest growth with a small bud, and a single, post-replicative nucleus at the bed neck. The tip of the nascent bud is the point of cell lysis. Currently, the most clearly understood function

for yeast Pkc1p is its role in activation of the Mpk1p MAP kinase cascade. It has been proposed that Pkc1p regulates additional pathways or processes since *pkc1Δ* mutants have more severe growth defects than mutants defective in subsequent steps in Mpk1p activation (Errede and Levin 1993).

Also referred to as the cell integrity pathway, the Mpk1p MAP kinase cascade consists of four proteins arranged in three tiers. The distal level of the signaling cascade is occupied by the Pkc1p substrate, Bck1p (Costigan *et al.* 1992, Lee and Levin 1992). *BCK1* encodes a 1478 amino acid residue protein kinase of the MAPKK kinase family. Bck1p activates a pair of homologous ~500 amino acid residue MAPK kinases encoded by the *MKK1* and *MKK2* genes (Irie *et al.* 1993). Mpk1p, a 484 amino acid residue protein kinase of the MAP kinase family, is the target of Mkk1p and Mkk2p phosphorylation (Lee *et al.* 1993). Activation of Mpk1p occurs in cells that have been exposed to high temperature, mating pheromones, or hypo osmotic shock (Martin *et al.* 1993, Buehrer and Errede 1997, Zarzov *et al.* 1996). In contrast to the HOG MAP kinase pathway, very little is known about negative regulatory elements that control signaling in the Mpk1p pathway. No serine/threonine or tyrosine phosphatases that dephosphorylate components of the cascade have been conclusively identified.

Several phenotypes, similar to those exhibited by *pkc1Δ* mutants, are common to mutants which lack activity in any one tier of the MAP kinase cascade. Cell lysis, due to defective cell wall construction, can be induced by incubating cells at high temperature, or in the presence of caffeine. This phenotype is suppressible by the addition of sorbitol to growth medium. Mpk1p pathway mutants also have actin cytoskeleton assembly deficiencies, are defective in the process of forming projections in response to mating pheromone, and are unable to grow on non-fermentable carbon sources such as glycerol.

1.8 Outputs From The Cell Integrity Pathway

Only a few direct effectors of transcription have thus far been identified as being modulated by Mpk1p. Rlm1p, a transcription factor with homology to the MADS (Mcm1, Agamous, Deficiens, Serum response factor) box family of transcription factors was identified during a screen for downstream targets of the cell integrity pathway where mutants were selected for their ability to bypass the growth-inhibitory effect of overactivating the MAP kinase pathway (Dodou and Triesman 1997, Watanabe *et al.* 1995). Rlm1p and Mpk1p interact in the two-hybrid system, and high copy expression of Rlm1p fused to the transcriptional activation domain of Gal4p was able to bypass the high temperature and caffeine sensitivities of *bck1Δ* and *mpk1Δ* mutants. Genes that are transcriptionally regulated by Rlm1p are presently not known.

The SBF transcription factor, composed of Swi4p and Swi6p, regulates expression of G1 cyclins, which when synthesized, go on to associate with the cyclin dependent kinases Cdc28p and Pho85p to promote entry into S-phase. Noting that *mpk1Δ* and SBF mutants had similar phenotypes, Madden *et al.* (1997) investigated whether SBF might be activated by Mpk1p. It was found that overexpression of *SWI4* and specific *PCL* cyclin genes could suppress several phenotypes of *mpk1Δ* mutants. Furthermore, phosphorylation of Swi6p is dependent on the presence of *MPK1*, and correlates with Mpk1p activity in vivo. Finally, in vitro assays have demonstrated that Mpk1p can directly phosphorylate both Swi4p and Swi6p.

SBF was shown to have a role in cell wall synthesis by Igual *et al.* (1996), who demonstrated that SBF mutants have weakened cell walls. The transcription of several cell wall genes (*FKS1*, *GAS1*, *KRE6*, *MNN1*, *VAN2*, and *CHS3*) is periodic, and is regulated by SBF. In the same work, it was established that transcription of *FKS1*, *MNN1*, and *CHS3* is also heavily dependent on Pkc1p and Mpk1p in a manner that is independent of the SBF complex. Essentially it appears that the cell wall integrity pathway affects transcription of

cell wall synthesis genes through SBF and another, presently unknown transcriptional regulator.

1.9 Upstream Regulators Of The Cell Integrity Pathway

Rho1p is a member of the small GTP-binding protein family which includes the RHO, RAC and CDC42 subfamilies (Madaule *et al.* 1987). Several key roles in cell wall biosynthesis have been ascribed to Rho1p. Firstly, the β-1,3-glucan synthase catalytic subunit physically interacts with Rho1p, and this interaction is necessary for the activation of β-1,3-glucan synthesis (Qadota *et al.* 1996, Drgnova *et al.* 1996). Secondly, Rho1p interacts with and activates Pkc1p, resulting in activation of the Cell Wall Integrity pathway (Kamada *et al.* 1996, Drgnova *et al.* 1996). A third link between Rho1p and the cell wall was established with the recent discovery that Rho1p interacts with Skn7p (Alberts *et al.* 1998). Like *pkc1Δ* mutants, cells depleted for Rho1p arrest growth with a small bud, which then lyses at the tip. However, this phenotype is not reversible by osmotic stabilizers, probably because Rho1p performs several functions in addition to activating Pkc1p .

Besides its vital role in cell wall assembly and organization, *RHO1* is required for cytoskeleton assembly. Rho1p interacts with the formin-like proteins Bni1p and Bnr1p which are required for a number of cytoskeletal-dependent processes (Imamura *et al.* 1997). Mutants lacking all Rho1p activity contain an apparently normal actin cytoskeleton after cell death. However, specific temperature-sensitive alleles of *RHO1* (*rho1-2*^{ts} and *rho1-5*^{ts}) cease growth with abnormal actin distribution and aberrant cell morphology when shifted to the nonpermissive temperature (Helliwell *et al.* 1998). Cytoskeletal defects are likely not seen in cells completely lacking Rho1p activity because they lyse due to cell wall defects before cytoskeletal abnormalities are manifested.

To enable interaction with its protein targets, RHO-like proteins typically have to be in the GTP bound state. When complexed with GDP, RHO proteins are usually inactive.

Switching between GTP and GDP-bound states is tightly regulated, and is facilitated by a several different proteins. The exchange of GDP for GTP is enabled by a set of proteins collectively known as GDP-GTP Exchange Factors (GEFs). The hydrolysis of GTP to GDP by the RHO protein is facilitated by GTPase-Activating Proteins (GAPs). Overall activity of RHO proteins is determined by the balance between the competing activities of the GAPs and GEFs. Mutations that abolish GEF activity strand the RHO protein in an inactive, GDP-bound state. Conversely, mutations that eliminate GAP activity result in a constitutively active, GTP-complexed protein.

The products of the *ROM1* and *ROM2* genes have been demonstrated to supply GEF activity towards Rho1p (Ozaki *et al.* 1996). *ROM1* and *ROM2* respectively encode 1155 and 1356 amino acid proteins that are ~50% identical to one another. Both proteins contain pleckstrin homology (PH) and Dbl-homologous (DH) domains, which are characteristic for RHO-type GEFs. *ROM2* apparently encodes the major Rho1p GEF activity since *rom1Δ* mutants do not have vegetative growth defects while *rom2Δ* mutants grow slowly, are sensitive to high temperature, and caffeine. In addition, *rom2Δ* cells are unable to form normal mating projections when exposed to mating pheromone (Manning *et al.* 1997). Mutants lacking both *ROM1* and *ROM2* are inviable at any temperature, even in the presence of osmotic support.

The product of the *SAC7* gene provides GAP activity towards Rho1p (Schmidt *et al.* 1997). *SAC7* was first isolated as an extragenic suppressor of a temperature sensitive actin mutant (*act 1-4*). At low temperatures, *sac7Δ* mutants have strongly reduced viability, and are disrupted in actin cytoskeleton assembly (Dunn *et al.* 1990). Presently, it is not known if *sac7Δ* mutants are affected in cell wall assembly.

TOR2 encodes a putative phosphotidyl inositol (PI) kinase that has two roles in cell physiology. One function is redundant with Tor1p, and is necessary for activation of translation initiation in response to nutrient availability (Barbet *et al.* 1996, Kunz *et al.* 1993). The second function is unique to Tor2p, and is required for cell viability and the cell

cycle dependent polarization of actin (Schmidt *et al.* 1996). An allele of Tor2p (*tor2*^{ts}) that loses only the Tor2p-unique function at elevated temperature (37°C) has been generated, and has been used to identify downstream targets of Tor2p. Overexpression of *ROM2* suppresses the inviability of *tor2*^{ts} mutants at high temperature, suggesting that Tor2p might function upstream of Rom2p (Schmidt *et al.* 1997). No physical interaction between Rom2p and Tor2p was detected in two hybrid assays, suggesting that the two proteins do not physically interact. However, the presence of a PH domain adjacent to the GEF domain in Rom2p suggests another way in which Tor2p might regulate Rom2p activity. PH domains have been suggested to be important for activation of signaling molecules, and have been shown to bind to PI derivatives (Lemmon *et al.* 1996). The observation that the PH domain of Rom2p is required for high copy *ROM2* to bypass the inviability of *tor2*^{ts} mutants at 37°C led Schmidt *et al.* (1996) to suggest that the PI kinase activity of Tor2p produces a phosphorylated phosphoinositide that binds to the Rom2p PH domain, resulting in Rom2p activation, and consequently Rho1p activation. However, this hypothesis remains to be verified by biochemical means.

Bickle *et al.* (1998) demonstrated the existence of a second pathway for activating Rho1p via Rom2p that is independent of Tor2p. It was observed that mutations in cell wall synthesis genes, or the addition of SDS to culture medium could suppress the inviability of *tor2*^{ts} mutants at the nonpermissive temperature. Suppression of the *tor2*^{ts} mutant by cell wall disturbances requires the presence of *ROM2*. Furthermore, it was demonstrated that perturbation of the cell wall stimulates Rho1p GEF activity. Since cell wall disturbances can bypass the *tor2*^{ts} mutation, presumably by activating Rho1p GEF activity, it was proposed that a second cell wall responsive mechanism, parallel to Tor2p. stimulates Rho1p GEF activity via Rom2p.

Recently, new proteins that influence activity in the cell integrity pathway have been identified. While screening for High Copy Suppressors (*HCS*s) of an **a**/α *swi4*^{ts}/*swi4Δ*

strain cultured at high temperature, Gray *et al.* (1997) isolated a gene they named *HCS77* (also known as *SLG1/WSC1*, and will be referred to as *WSC1*). The *WSC1* open reading frame encodes a predicted protein of 378 amino acid residues in length. Hydropathy plot analysis of Wsc1p sequence reveals an N-terminal signal sequence and a single membrane-spanning region that occurs in the C-terminal third of the protein. These two features together suggest that Wsc1p adopts a Type I conformation (N-terminus is extracellular, single TMD, and cyoplasmic C-terminus), and is targeted to the plasma membrane. Confirming these predictions, a Wsc1p-GFP fusion protein localizes to the plasma membrane, and is uniformly distributed throughout it. Between the signal peptide sequence and transmembrane domain, the sequence of Wsc1p is rich in serine and threonine residues, potential sites of O-linked mannosylation.

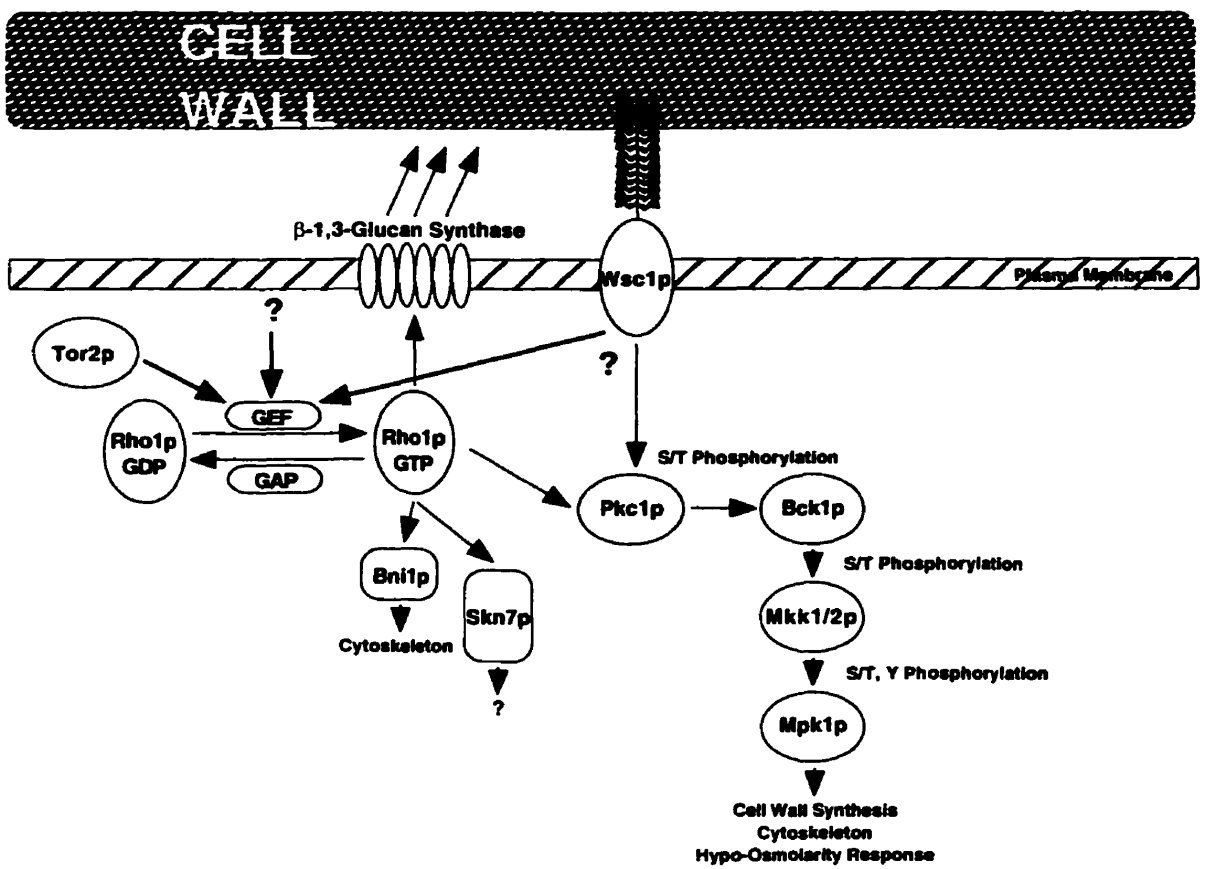
There are two *S. cerevisiae* genes that encode functional homologs of Wsc1p, *WSC2* and *WSC3* (Verna *et al.* 1997). Overall, the three *WSC* family members are 50% similar and 35% identical to each other, with Wsc2p and Wsc3p showing the highest degree of sequence conservation. Wsc2p and Wsc3p share the predicted sequence characteristics of Wsc1p, including the N-terminal signal sequence, the serine/threonine-rich region, and the single transmembrane domain. In addition, sequence analysis of the *WSC* trio revealed the existence of a cysteine motif located immediately C-terminal to the signal peptide sequence. Cysteine motifs have been implicated in ligand binding, dimerization, and zinc ion-coordination processes.

Cells lacking *WSC1* are slow growing, prone to lysis, and sensitive to high temperature and caffeine. *wsc1Δ/wsc1Δ* diploids shifted from 25°C to 37°C arrest growth as small-budded cells with a DNA content between 1N and 2N. Although *wsc2Δ* or *wsc3Δ* mutants on their own do not have discernable growth and viability phenotypes, mutants lacking *WSC1* and *WSC2* and/or *WSC3* have stronger phenotypes than cells lacking only *WSC1*.

Several lines of evidence together suggest that WSC proteins function as positive regulators of the cell wall integrity pathway and occupy a distal position in the signaling cascade hierarchy. Firstly, the phenotypes displayed by *wscΔ* mutants are highly reminiscent of those manifested in cell wall integrity pathway mutants. Secondly, *wsc1Δ* mutants are sensitive to small losses in Pkc1p activity, and high copy *WSC1* can not suppress mutants lacking Pkc1p or members of the MPK1 MAP kinase cascade. Thirdly, high copy plasmids bearing components of the *PKC1-MPK1* pathway, including *PKC1*, *BCK1-20* (a hyperactive allele), *STE20*, *MPK1*, *ROM1* and *ROM2* could suppress the caffeine sensitivity of *wsc1Δ* mutants (Jacoby *et al.* 1998). Finally, activation of Mpk1p in response to high temperature stress is attenuated in the absence of WSC family members.

Figure 1.3 Model of the cell integrity pathway. Currently known interactions between the members of the cell integrity pathway are summarized.

The Cell Integrity Pathway



1.10 Outline of Experimental Approach

To verify predictions that Skn7p is regulated by a histidine kinase, I used a reporter system that measures the transcriptional activation potential of Skn7p. By assaying Skn7p activity in different genetic backgrounds, I was able to determine that Skn7p is regulated by the Sln1p two-component histidine kinase. Since Sln1p's only known function is to regulate signaling in the osmoresponsive MAP kinase cascade, I then examined the possibility that Skn7p also plays a role in regulating the high osmolarity response. The results from these experiments are presented in Chapter 2. In an earlier attempt to identify the upstream regulators of Skn7p, the gene encoding Mid2p was isolated as a multicopy activator of Skn7p. Using a number of biochemical and genetic approaches, I examined the physical characteristics of Mid2p, and defined a role for it in the regulation of cell integrity responsive MAP kinase cascade. Chapter 3 contains the results of these experiments. Chapter 4 describes the results obtained from genetic experiments and GDP-GTP exchange assays for Rho1p performed in order to test the hypothesis that Mid2p is an upstream regulator of Rho1p.

CHAPTER 2

Yeast Skn7p Activity is Modulated by the Sln1p-Ypd1p Osmosensor and Contributes to Regulation of the HOG Pathway

2.1 ABSTRACT

Activation and control of the yeast HOG (High Osmolarity Glycerol) MAP kinase cascade is accomplished, in part, by a two-component sensory-response circuit comprised of the osmosensing histidine protein kinase Sln1p, the phospho-relay protein Ypd1p, and the response regulator protein Ssk1p. We found that deletion of *SLN1* and/or *YPD1* reduces reporter gene transcription driven by a second two-component response regulator; Skn7p. The effect of *sln1Δ* and *ypd1Δ* mutations upon Skn7p activity is dependent on a functional two-component phosphorylation site (D427) in Skn7p, suggesting that Sln1p and Ypd1p may act as phosphodonors for Skn7p. We also observed that loss of *PTC1* (a protein serine/threonine phosphatase implicated in negative control of the HOG pathway) in a *skn7Δ* background results in severe slow growth and morphological defects. Deletion of either *PBS2* or *HOG1* alleviates the slow growth of *ptc1Δ skn7Δ* cells, suggesting that Skn7p may participate in concert with known regulatory components in modulating HOG pathway activity. The contribution of Skn7p to HOG pathway regulation appears to be modulated by the receiver domain since unphosphorylatable Skn7p^{D427N} is unable to fully restore growth to *ptc1/skn7* cells.

2.2 Introduction

Although numerous histidine protein kinase-response regulator pairs comprising distinct sensory-response systems can be found in bacteria such as *Escherichia coli*(Parkinson and Kofoid 1992), *S. cerevisiae* does not appear to make extensive use of the two-component signaling circuit. It has only one histidine protein kinase homolog (Sln1p) and two response regulator protein homologs: Ssk1p, and Skn7p, a protein that appears to be involved in several distinct processes including cell wall assembly (Brown *et al.* 1993; Brown *et al.* 1994), oxidative stress response (Krems *et al.* 1996; Morgan *et al.* 1997), and G1 cyclin gene expression (Morgan *et al.* 1995). While a small proportion is found in the cytoplasm, Skn7p is predominately localized to the nucleus and contains a region homologous to the DNA-binding domain of heat shock transcription factors as well as a domain with significant homology to the receiver domain of two-component response regulators (Brown *et al.* 1993), including a conserved aspartic acid (D427) that receives phosphorylation from cognate histidine kinases. Skn7p appears to function as a transcription factor based on demonstration of its ability to bind to promoter elements (Morgan *et al.* 1997) and to activate transcription of reporter genes (Brown *et al.* 1994). This ability to activate transcription is influenced by mutations at the phosphorylation site (D427) of the Skn7p receiver domain, and this finding led to predictions that a histidine protein kinase directs phosphorylation of Skn7p (Brown *et al.* 1994). However, the identity of this kinase remained unknown. We investigated the possibility that Skn7p activity is influenced by the osmosensing histidine kinase Sln1p and its partner Ypd1p. We also explored a possible regulatory role for Skn7p in the osmotic stress signaling pathway.

2.3 Materials and Methods

Reporter Assays

Measurement of transcriptional activation of the lacZ reporter gene was performed in strain L40 [*MATa his3 trp1 leu2 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4*] (gift of S. Hollenberg), essentially as described [Dolan and Fields 1990; Brown *et al.* 1994). Cells grown in selective medium were diluted into low-osmolarity (YEPD) medium and grown eight hours before harvesting. ß-galactosidase activity was calculated (where t=reaction time in minutes, v=volume of cells used in reaction, d=dilution factor of OD_{600} reading) as $(1000 \times OD_{420} \times d)/(t \times OD_{600})$.

Construction of Strains

The *SSK1* gene was disrupted in L40 with the *KANMX2* module by PCR-based gene disruption (Wach *et al.* 1994). The *SLN1* gene was disrupted in *ssk1Δ* cells with the *GFP-HIS3* cassette by PCR-based gene disruption (Niedenthal *et al.* 1996). The *YPD1* gene was disrupted in *ssk1Δ* and *ssk1Δ sln1Δ* cells with the *ADE2* gene by PCR-based gene disruption. All gene disruptions were verified by PCR using specific primers.

Analysis of genetic interactions between *SKN7* and various genes of the HOG pathway was performed in the SEY6210 strain. Gene disruption was carried out in the SEY6210 diploid strain [*MATα/MATa leu2-3,112/leu2-3,112 ura3-52/ura-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ90/trp1-Δ90 suc2-Δ9/suc2-Δ9*]. Mutant haploids were obtained by sporulation of diploids and tetrad dissection. Diploid mutants heterozygous for more than one gene were obtained by mating of appropriate haploid mutants. The *HOG1*, *PBS2*, *PTC1*and *PTP2* genes were disrupted with the *KANMX2* module. Each gene disruption

was verified by PCR with specific primers. Disruption of *SKN7* is described in Brown *et al.* 1993, and disruption of *PTC1* by *URA3* is described in Jiang *et al.* 1995.

All tetrad dissections were performed on YEPD agar plates which were subsequently incubated at 30°C for 72 hours before photographing. All genotypes were determined by replica-plating onto required selective media, and by PCR analysis of colonies with specific primers.

2.4 Results

Skn7p Activation Requires Sln1p and Ypd1p

We produced the fusion protein LexA-Skn7p and measured its influence on (lexAop)8-driven LacZ expression in the yeast reporter strain L40 (Brown *et al.* 1994). We examined the effect of deleting *SLN1*, *YPD1* or both genes on LexA-Skn7p-dependent reporter activation to determine if they are required for Skn7p activity. Deletion of *SLN1* and/or *YPD1* causes excessive stimulation of the HOG pathway via hyperactive Ssk1p, and results in extreme growth defects or lethality depending on medium composition and strain background (Ota and Varshavsky 1993; Maeda *et al.* 1994), therefore it was necessary to also delete *SSK1*. Compared to the activity observed in wild type L40, LexA-Skn7p-driven reporter activity was significantly lower in *ssk1*Δ *sln1*Δ, *ssk1*Δ *ypd1*Δ, and *ssk1*Δ *sln1*Δ *ypd1*Δ strains (Fig. 2.1A). Deletion of *SSK1* alone had little or no effect on expression of the reporter gene. We also investigated whether contribution of Sln1p and Ypd1p to LexA-Skn7p activity required an intact aspartic acid phosphorylation site in the Skn7p receiver module. Fusion proteins LexA-Skn7p^{D427N} and LexA-Skn7p^{D427E} were expressed in wild type, *ssk1*Δ, *ssk1*Δ *sln1*Δ, *ssk1*Δ *ypd1*Δ, and *ssk1*Δ *sln1*Δ *ypd1*Δ L40 reporter strains. Previous work had demonstrated that the D427N mutation diminished the activity of Skn7p and that the D427E mutation resulted in hyper activation of Skn7p (Brown *et al.* 1994).

Our results indicate that LexA-Skn7p^{D427N}-driven reporter activity is the same low level in each of the five reporter strains tested (Fig. 2.1B). This low level is approximately the same as that observed with LexA-Skn7p in the *ssk1*Δ *sln1*Δ, *ssk1*Δ *ypd1*Δ, and *ssk1*Δ *sln1*Δ *ypd1*Δ strains. Reporter activity driven by LexA-Skn7p^{D427E} was unaffected by deletion of *SLN1* and/or *YPD1*, and was observed to be roughly four fold higher than that seen in LexA-Skn7^{D427N} (Fig. 2.1C). Taken together, the results of Fig. 2.1 indicate that Sln1p and Ypd1p are required for the D427-dependent activity of Skn7p as a transcriptional activator.

Genetic Interactions Between SKN7 and Members of the HOG pathway

In light of the observation that Skn7p activity appears to be, at least partially, regulated by the Sln1p osmosensor, we addressed the possibility that Skn7p is involved in the osmotic stress response pathway. Compared to wild type cells, *skn7Δ* cells are not altered in their ability to grow on hyperosmotic media. However, overexpression of *SKN7*^{D427E} results in mildly diminished growth on high salt media, suggesting that Skn7p might in some way be involved in osmotolerance (Fig. 2.2A). With this possibility in mind we searched for genetic interactions between *SKN7* and various members of the HOG pathway. We found that *skn7*Δ *ptc1*Δ mutants display a severe slow growth phenotype (Fig. 2.2B). Slow colony growth is also characteristic of *ptc1Δ/ptp2Δ* cells in which phosphorylation by kinases of the HOG MAP kinase cascade is deregulated.

Figure 2.1 Requirement for Sln1p and Ypd1p for Skn7p activation of transcription. Wild type and mutant L40 reporter strains carrying (**A**)*LEXA-SKN7*, (**B**)*LEXA-SKN7*^{D427N}, (**C**)*LEXA-SKN7*^{D427E}, (**D**)pBTM116 only. Level of ß-galactosidase activity is represented on the Y-axis (note smaller scale in (**C**) to accommodate higher activity levels), and reporter genotype is represented on the X-axis.

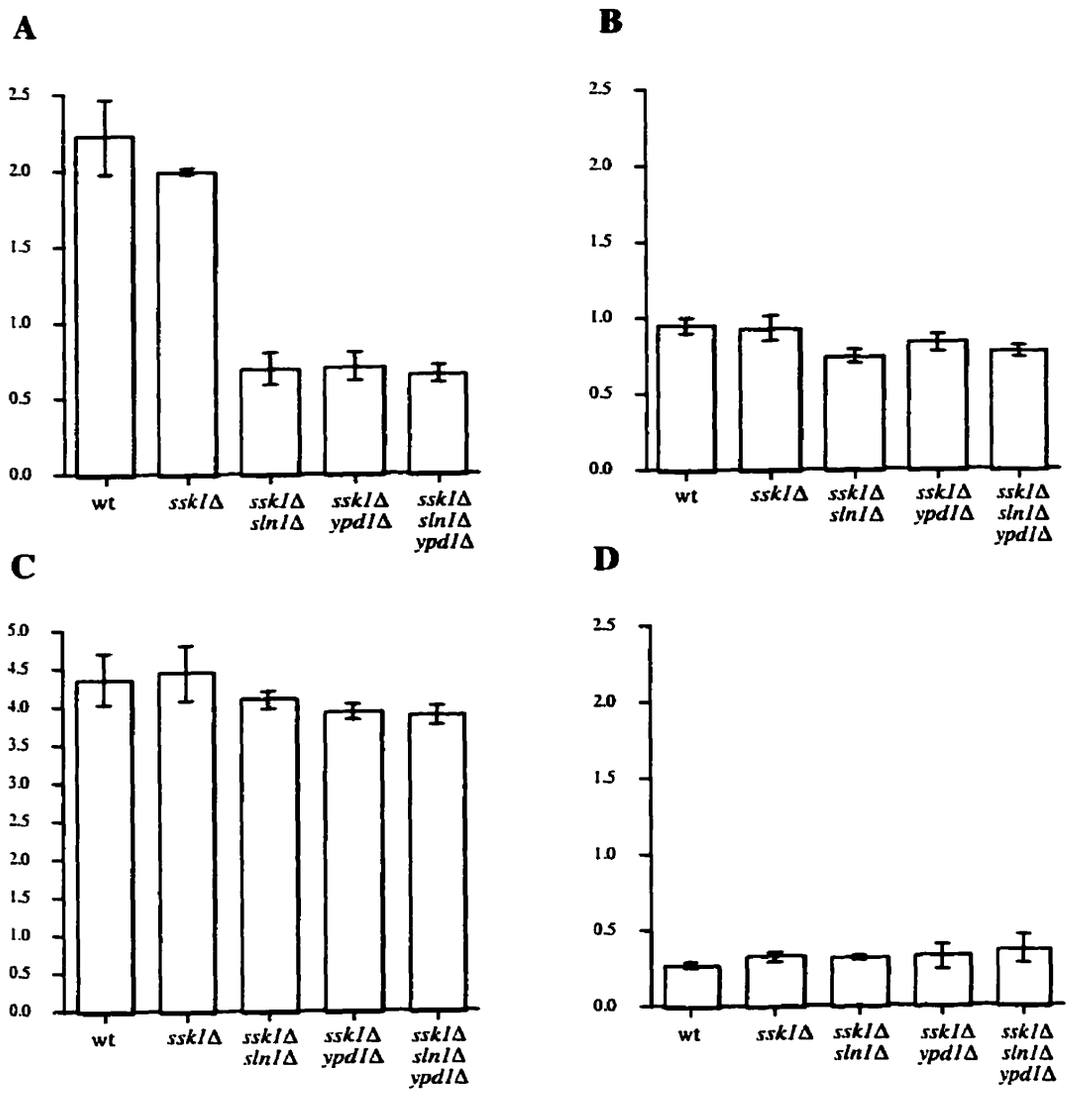
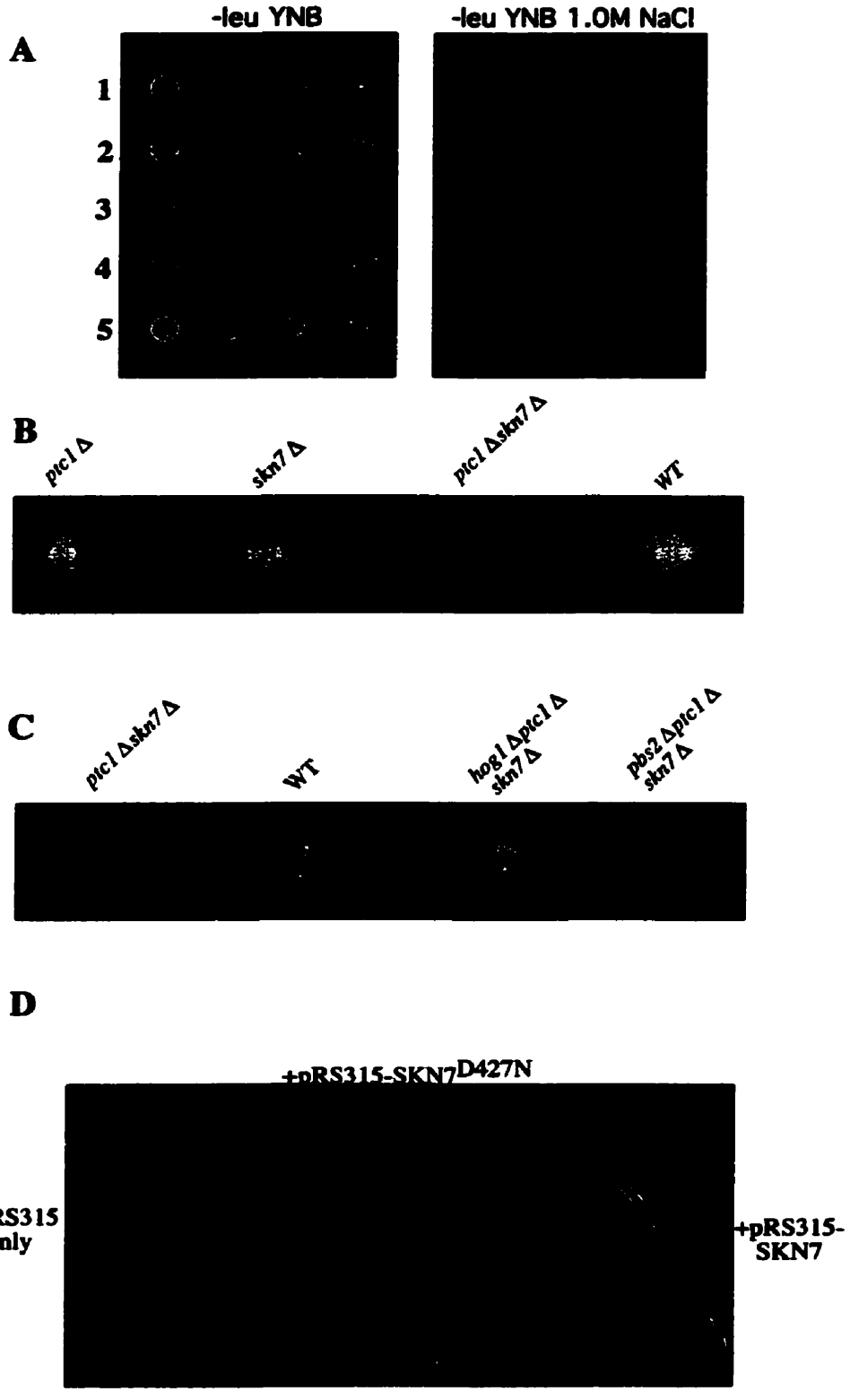

A
2.5
2.0
1.5
1.0
0.5
0.0
wt
sskl Δ
sskl Δ sln1 Δ
sskl Δ ypd1 Δ
sskl Δ sln1 Δ ypd1 Δ
B
2.5
2.0
1.5
1.0
0.5
0.0
wt
sskl Δ
sskl Δ sln1 Δ
sskl Δ ypd1 Δ
sskl Δ sln1 Δ ypd1 Δ
C
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
wt
sskl Δ
sskl Δ sln1 Δ
sskl Δ ypd1 Δ
sskl Δ sln1 Δ ypd1 Δ
D
2.5
2.0
1.5
1.0
0.5
0.0
wt
sskl Δ
sskl Δ sln1 Δ
sskl Δ ypd1 Δ
sskl Δ sln1 Δ ypd1 Δ

Figure 2.2 Skn7p contributes to HOG pathway regulation. (**A**) Effect of high osmolarity on strains overexpressing *SKN7*. Log phase cultures of cells were grown in selective medium, diluted to an OD_{600} of 0.1 then 3ul each of consecutive serial dilutions were spotted onto selective media of low and high osmolarity. The genotypes of the cells are **1)** wild type +pRS425 (2μ *LEU2*), **2)** *skn7Δ*+pRS425, **3)** *skn7Δ*+pRS425-$SKN7^{D427E}$ **4)** *skn7Δ*+pRS425-*SKN7* **5)** *skn7Δ*+pRS425-$SKN7^{D427N}$(**B**) Tetrad from dissection of a heterozygous diploid *ptc1Δ skn7Δ* -derived ascus. (**C**) Representative colonies obtained by dissection of asci demonstrating the HOG pathway-dependent growth defect of *ptc1Δ skn7Δ* mutants. (**D**) Ability of $SKN7^{D427N}$ to restore growth to *ptc1Δ skn7Δ* cells. *ptc1Δ skn7Δ* cells carrying pRS316-*PTC1* were transformed with pRS315 (Cen *LEU2*), pRS315-*SKN7* and pRS315-$SKN7^{D427N}$. LEU+ tranformants were streaked on to LEU^- agar containing 5-fluoroorotic acid to select cells that had lost pRS316-*PTC1*. Colonies of each genotype were then streaked on to LEU^- agar, and incubated at 30°C for 36 hours.


A
-leu YNB
-leu YNB 1.0M NaCl
1
2
3
4
5
B
ptc1 Δ
skn7 Δ
ptc1 Δskn7 Δ
WT
C
ptc1 Δskn7 Δ
WT
hog1 Δptc1 Δ skn7 Δ
pbs2 Δptc1 Δ skn7 Δ
D
+pRS315-SKN7D427N
+pRS315 only
+pRS315-SKN7

Normal growth can be restored to *ptc1Δ/ptp2Δ* cells by deletion of *HOG1* or *PBS2*. We blocked HOG pathway signaling in *ptc1Δ skn7Δ* cells by deletion of either *PBS2* or *HOG1*, and observed that the triple mutants *hog1Δ ptc1Δ skn7Δ* and *pbs2Δ ptc1Δ skn7Δ* were restored for growth rate(Fig. 2.2C), implying that slow growth in *ptc1Δ skn7Δ* cells may be due to unregulated activity in the HOG pathway. This possibility is also supported by two other lines of evidence. Firstly, we found that mutants lacking both *PTC1* and *SKN7* had higher intracellular glycerol levels than wild type cells or cells lacking either *PTC1* or *SKN7* (Fig. 2.3). Secondly, we isolated the *PTP2* and *PTC2* phosphatase genes in a genetic screen designed to identify multicopy suppressors of slow growth in *ptc1Δ skn7Δ* cells (not shown).

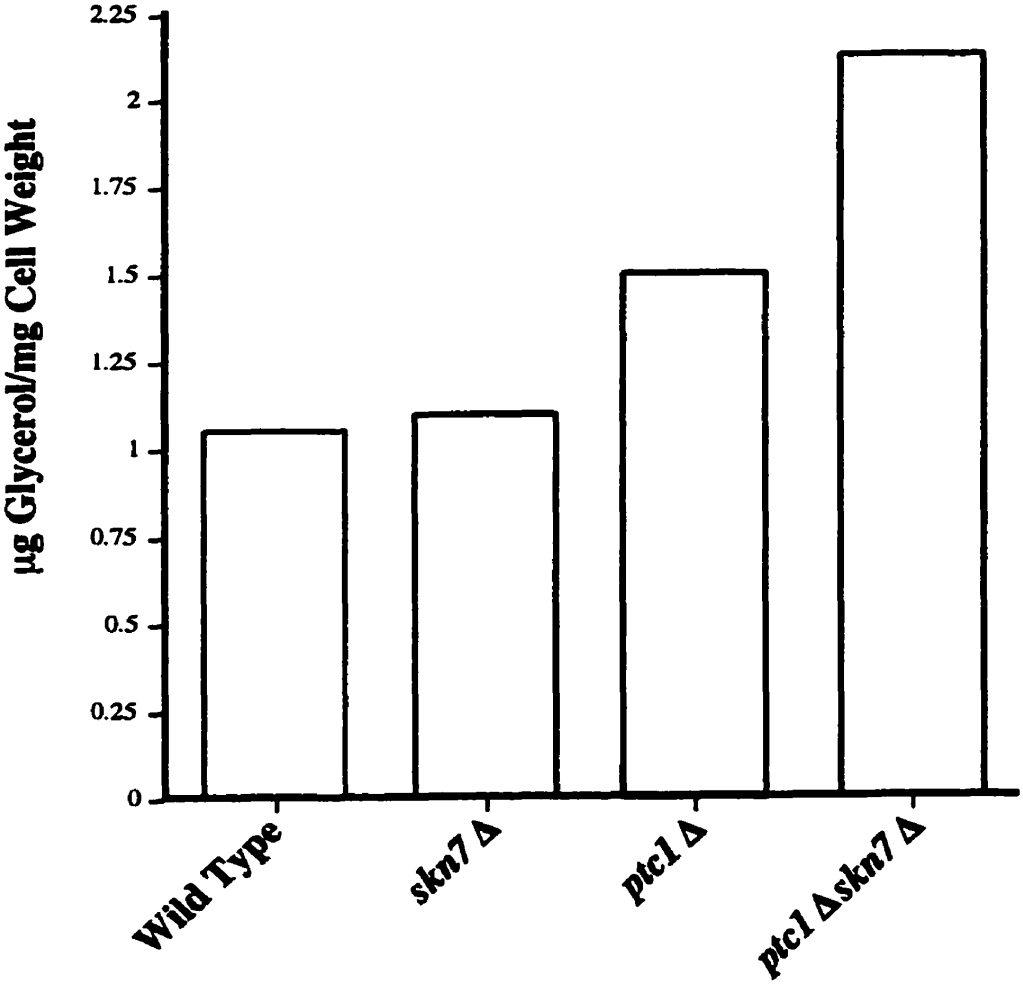
Although *ptp2Δ skn7Δ* cells do not display a synthetic growth defect, the triple mutant *ptc1Δ ptp2Δ skn7Δ* is inviable (not shown). Spores from this mutant are able to germinate and form microcolonies but cease to grow after a small number of cell divisions. The two quadruple mutants *hog1Δ ptc1Δ ptp2Δ skn7Δ* and *pbs2Δ ptc1Δ ptp2Δ skn7Δ* grow well, suggesting that inviability in the *ptc1Δ ptp2Δ skn7Δ* mutants is due to further deregulation of signaling events in the HOG pathway. The synthetic interactions between *SKN7*, *PTC1*, and *PTP2* again suggest that, like Ptc1p and Ptp2p, Skn7p contributes to HOG pathway regulation.

Requirement for a Functional Receiver Domain in Skn7p

To determine if a functional receiver domain is required for Skn7p activity in HOG pathway regulation, we examined the ability of unphosphorylatable Skn7p^{D427N} to suppress the growth defect of *ptc1Δ skn7Δ* cells (Fig. 2.2D). We found centromeric plasmid-borne *SKN7*^{D427N} to be better at suppression than a vector alone but less capable than *SKN7* of

restoring growth to *ptc1Δ skn7Δ* cells. This observation correlates well with our finding that LexA-Skn7p-mediated transcriptional activation is significantly diminished but not completely eliminated by the D427N mutation. Thus, signaling via the receiver domain modulates the contribution of Skn7p to HOG pathway regulation. In contrast to its requirement in HOG pathway regulation, D427 does not appear to be required for Skn7p to function in the oxidative stress response (Morgan *et al.* 1997). Cells containing $SKN7^{D427N}$ are not hypersensitive to H_2O_2 (Morgan *et al.* 1997) and cells containing $SKN7^{D427E}$ are not more resistant to H_2O_2 than wild type cells (not shown). Additionally, we found that the mutants *ssk1Δ*, *ssk1Δ sln1Δ*, *ssk1Δ ypd1Δ* and *ssk1Δ sln1Δ ypd1Δ* are not sensitive to H_2O_2 stress (not shown), suggesting that the other yeast two-component proteins are not involved in the oxidative stress response. This observation suggests that with respect to oxidative stress, Skn7p is likely to be regulated by another pathway.

Figure 2.3 Intracellular Glycerol concentration of cells. Intracellular concentration of glycerol was determined using a glycerol assay kit (Boehringer Manheim). Cells were grown to mid-log phase in YEPD liquid medium, harvested, washed once, weighed and processed according to kit instructions.

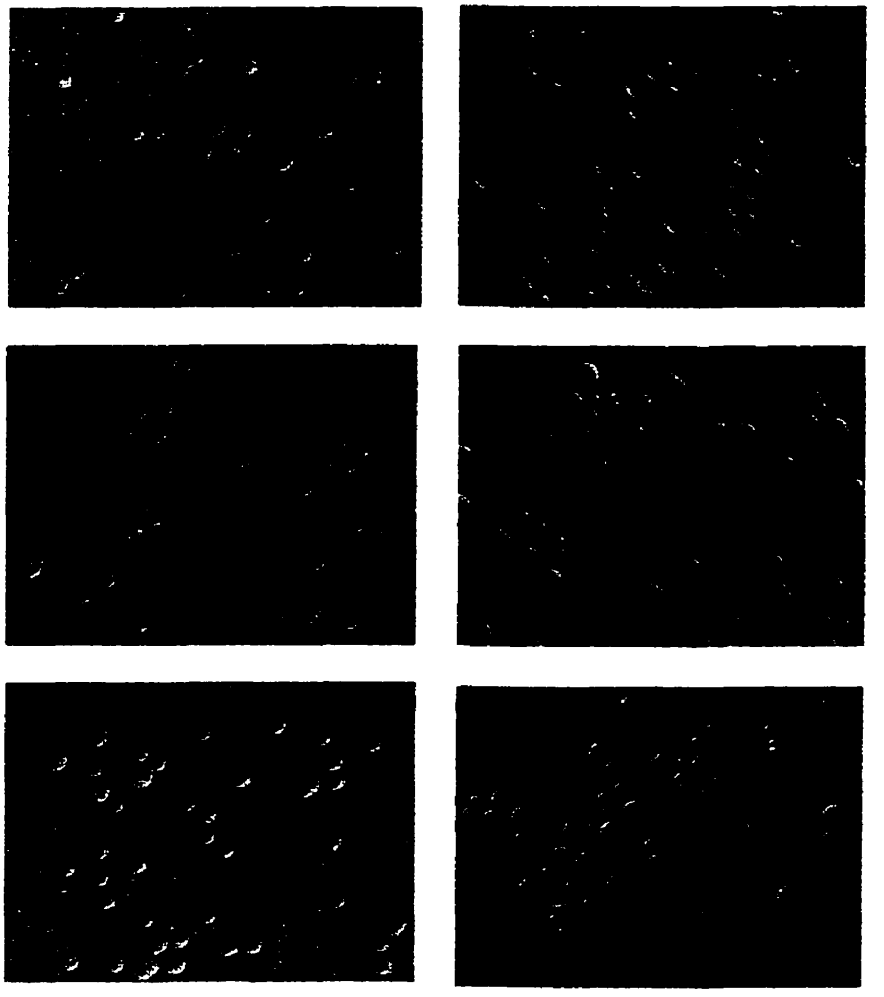

µg Glycerol/mg Cell Weight
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
Wild Type
skn7 Δ
ptc1 Δ
ptc1 Δskn7 Δ

Morphological Defects in Mutants

Microscopic examination revealed that *ptc1Δ skn7Δ* cells display strong morphological defects. Many cells display multiple buds and/or elongated, deformed buds (Fig. 2.4D). Significantly, although *hog1Δ ptc1Δ skn7Δ* mutants are restored for growth rate, many of these cells still exhibit abnormal morphology (Fig 2.4F). In contrast, *pbs2Δ ptc1Δ skn7Δ* mutants do not manifest morphological defects (Fig. 2.4E). These observations suggest that for *ptc1Δ skn7Δ* cells, slow growth stems from effects downstream of Hog1p while morphological anomalies are likely to be largely the result of Pbs2p activating Hog1p-independent substrate(s) or pathway(s) that influence cell morphology.

The phosphatases Ptc1p, Ptc3p, Ptp2p and Ptp3p act directly on Pbs2p and Hog1p, while Skn7p, as a transcription factor, possibly exerts its influence indirectly by regulating the level of some inhibitor of the HOG pathway (e.g., a phosphatase, kinase or other protein that serves to inhibit the pathway) . We explored the possibility that Skn7p was involved in transcriptional control of phosphatase gene(s) known or suspected to regulate the HOG pathway. Northern blot analysis indicated that *skn7Δ* cells were not significantly reduced compared to isogenic wild type cells for *PTC1*, *PTC2*, *PTC3*, *PTP1*, *PTP2*, and *PTP3* mRNA transcripts (not shown). Therefore, the presumed transcriptional target of Skn7p involved in HOG pathway regulation remains unknown.

Figure 2.4 Morphological defects of mutants. Wild type (**A**), *ptc1Δ* (**B**), *skn7Δ* (**C**), *ptc1Δ skn7Δ* (**D**), *pbs2Δ ptc1Δ skn7Δ* (**E**), and *hog1Δ ptc1Δ skn7Δ* (**F**) cells were grown on YEPD agar at 30°C for 48 hours. Cells were fixed in 3.7% formaldehyde 0.15M NaCl buffer for photography.

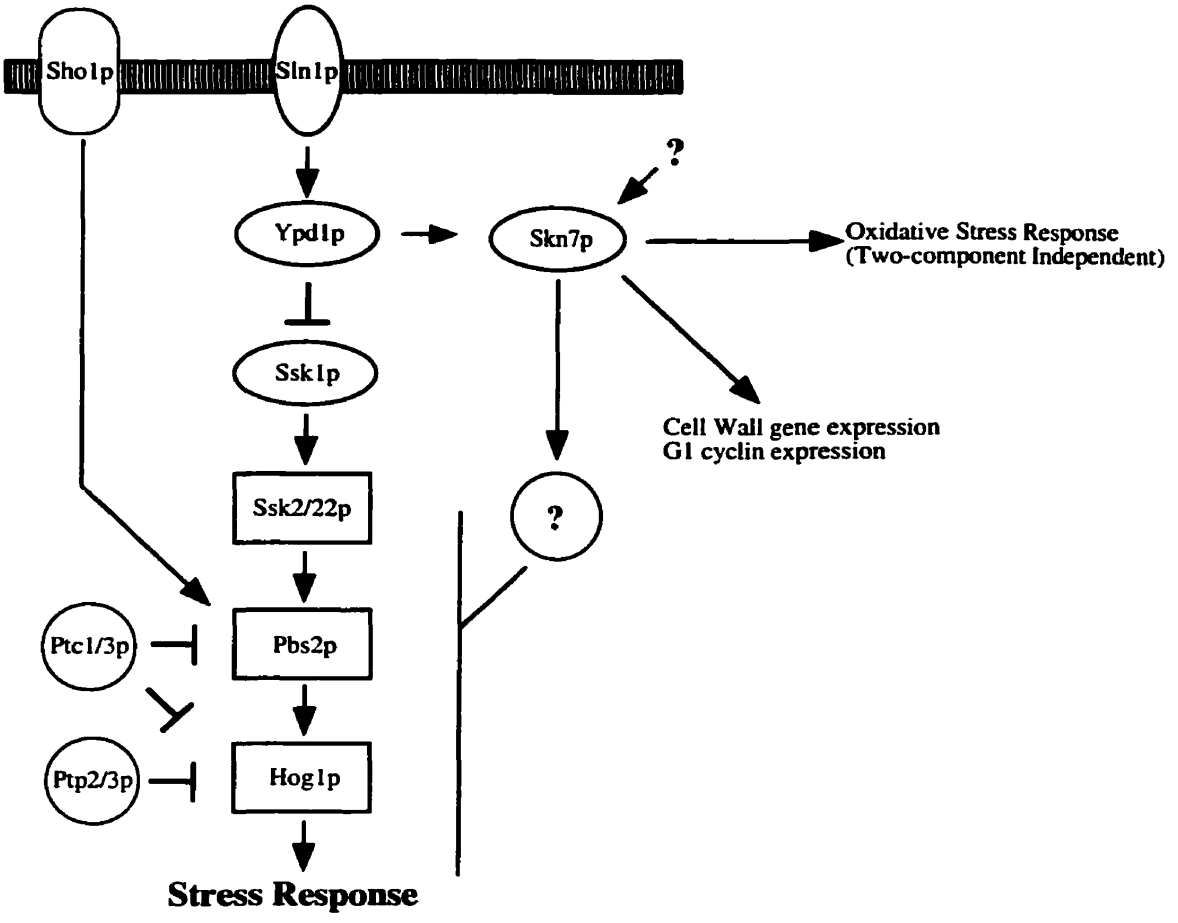


2.5 Discussion

Our findings indicate that the two-component sensor protein kinase Sln1p and its accessory phospho-relay protein Ypd1p are required to stimulate Skn7p transcriptional activity. Thus, all four of the identified *S. cerevisiae* two-component protein homologs participate collectively in a "four-component" signal transduction pathway (Fig. 2.5). The existence of two response regulator proteins, Ssk1p and Skn7p, receiving information from a single kinase/phospho-relay (Sln1p/Ypd1p) suggests that a branch point exists. Such a branch point provides two distinct ways for the system to modulate activity of the HOG signal transduction pathway: phosphorylation of Ssk1p serves to prevent activation of Ssk2p and Ssk22p kinase activity, and phosphorylation of Skn7p stimulates activity counteractive to the HOG pathway. This situation exhibits an intriguing similarity to the branch point in the two-component signaling pathway that enables *E. coli* to accomplish chemotaxis. In this bacterial system the sensor kinase directs phosphorylation of two different response regulator proteins, one that results in downstream activation and one that sets into motion events that dampen signaling and enable adaptation (Stock and Surette 1996).

The assay we employed does not directly demonstrate phospho-transfer from Sln1p to Ypd1p to Skn7p. However, based on the following observations, our data strongly imply that phospho-transfer from Sln1p to Ypd1p to Skn7p is occurring. 1. Activation of Skn7p-mediated reporter activity relies on Sln1p and Ypd1p in a D427-dependent manner. 2. The conserved aspartic acid (D427 in Skn7p) has been demonstrated to be a phosphorylation acceptor site in several different proteins. 3. There are no other yeast genes that encode two-component kinase homologs. It would be predicted that exposure to osmotic stress, which results in diminished activity in Sln1p, would result in reduced Skn7p-dependent reporter transcription.

Figure 2.5 Model of the *S. cerevisiae* osmotic stress response system, including the "four-component" modulation of the HOG signal transduction pathway. Under low external osmolarity conditions, active Sln1p transfers phosphates to Ssk1p and Skn7p via Ypd1p. Aspartic acid phosphorylated Ssk1p is unable to activate the MAPKKKs Ssk2p/Ssk22p, and Skn7p transcriptional activity increases. External osmotic stress reduces Sln1p activity and unphosphorylated Ssk1p is able to activate Ssk2p/Ssk22p. In addition Sho1p activates Pbs2p. The pathway is regulated by phosphatases at the level of Pbs2p and Hog1p and by Skn7p at a yet to be identified junction.


Sho1p
Sln1p
?
Ypd1p
Skn7p
Oxidative Stress Response
(Two-component Independent)
Ssk1p
Cell Wall gene expression
G1 cyclin expression
Ssk2/22p
?
Ptc1/3p
Pbs2p
Ptp2/3p
Hog1p
Stress Response

We did detect a small drop in reporter activity (~15-20%) in cells harboring the wild type LexA-Skn7p fusion protein after exposure to 0.3M NaCl for one hour (not shown). We feel that the type of assay we employed, while suitable for measuring steady state and induced reporter expression, is not appropriate for measuring transient decreases in reporter expression due to the inherent stability of the reporter ß-galactosidase protein. A more sensitive means of assaying Skn7p activity in response to osmotic stress will have to be developed.

Curiously, the two-component proteins apart from Skn7p are not required for H_2O_2 resistance, and the conserved aspartic acid in the receiver domain of Skn7p is apparently dispensable for Skn7p's contribution to the oxidative stress response. This finding suggests that Skn7p participation in the oxidative stress response is directed by another means. It was noted by Brown et. al (1994), that Skn7p is phosphorylated in a D427-independent manner, possibly on serine, threonine and tyrosine resides. This two-component independent phosphorylation may provide another means for regulation of Skn7p activity.

It is significant to note that *skn7Δ* mutants do not display HOG pathway-related defects unless *PTC1* is also deleted. Single deletions of individual regulators of the HOG pathway do not result in substantial growth defects due to aberrant HOG pathway signaling. Growth defects appear only when two or more regulators are missing simultaneously. This suggests that control of the pathway is established by a complex network of regulators with overlapping activities. Some components can partially compensate for missing ones, until too many components are removed, as in the lethal combination of *ptc1Δ ptp2Δ skn7Δ* mutations.

It is not yet clear where Skn7p exerts its regulatory influence on the HOG pathway. One possibility is that it controls the transcription of some protein(s) that directly regulate signaling within the MAP kinase cascade itself. Another possibility is that Skn7p acts

below Hog1p by interacting with Hog1p-modulated transcription factors. We are currently addressing these possibilities by employing whole-genome transcript profiling in order to identify Skn7p-dependent transcripts.

Given that MAP kinases cascades appear to be a conserved method of eukaryotic signal transduction (Davis 1994; Herskowitz 1995; Waskiewicz and Cooper 1995) and that two-component homologs are present in eukaryotic species as diverse as budding and fission yeasts (Shieh *et al.* 1997), and higher plants (Chang *et al.* 1993), it is possible that such a multi-tiered two-component protein control of MAP kinase cascades is a widespread regulatory theme in eukaryotes.

CHAPTER 3

Saccharomyces cerevisiae Mid2p Is a Potential Cell Wall Stress Sensor and Upstream Activator of the *PKC1-MPK1* Cell Integrity Pathway

3.1 Abstract

The *MID2* gene in *Saccharomyces cerevisiae* encodes a protein with structural features indicative of a plasma membrane-associated cell wall sensor. *MID2* was isolated as a multicopy activator of the Skn7p transcription factor. Deletion of *MID2* causes resistance to calcofluor white, diminished production of stress-induced cell wall chitin under a variety of conditions and changes in growth rate and viability in a number of different cell wall biosynthesis mutants. Overexpression of *MID2* causes hyperaccumulation of chitin and increased sensitivity to calcofluor white. α factor hypersensitivity of *mid2Δ* mutants can be suppressed by overexpression of upstream elements of the cell integrity pathway, including *PKC1*, *RHO1*, *WSC1* and *WSC2*. Mid2p and Wsc1p appear to have overlapping roles in maintaining cell integrity since *mid2Δ wsc1Δ* mutants are inviable on medium that does not contain osmotic support. A role for *MID2* in the cell integrity pathway is further supported by the finding that *MID2* is required for induction of Mpk1p tyrosine phosphorylation during exposure to α factor, calcofluor white or high temperature. Our data are consistent with a role for Mid2p in sensing cell wall stress, and activation of a response that includes both increased chitin synthesis and the Mpk1p MAP kinase cell integrity pathway. In addition, we have identified an open reading frame, *MTL1*, which encodes a protein with both structural and functional similarity to Mid2p.

3.2 Introduction

The cell wall is an essential organelle in fungal species. In *Saccharomyces cerevisiae* it is composed of four polysaccharide polymer classes; ß-1,3-glucan, ß-1,6-glucan, mannan, and chitin. The functions provided by the yeast cell wall include the determination of cell shape, protection of osmotic integrity, and scaffolding for extracellular proteins important for nutrient uptake and agglutination between mating partners. Recent research has emphasized the dynamic nature of this structure (see Cabib *et al.* 1997 and Orlean 1997 for reviews), which undergoes major changes in shape and composition during the vegetative budding cycle and the alternative developmental pathways of mating and sporulation.

Stress can lead to alteration of polymer levels in the yeast cell wall. This effect is perhaps best documented for chitin. Schekman and Brawley (1979) noted that during shmoo formation, a process during which the cell wall is rapidly remodeled, additional chitin is synthesized and deposited at the base and neck region of the mating projection. Roncero and Duran (1985) noted that exposure to calcofluor white, a substance which binds primarily chitin in the yeast cell wall, interferes with proper wall synthesis and induces elevated chitin synthesis *in vivo*. Recently, Popolo *et al.* (1997) and Ram *et al.* (1998) observed that mutational disruption of cell wall biosynthesis caused increased chitin deposition. This finding led to the proposal that alterations in cell wall assembly cause yeast to engage a compensation mechanism that includes increased chitin synthesis.

The *PKC1-MPK1* signal transduction pathway plays an essential role in maintaining the integrity of the cell wall during both mating and vegetative growth. Igual *et al.* (1996) showed that at least part of this influence on cell wall construction is the result of control of transcription of a variety of genes involved in cell wall biosynthesis. Pkc1p, a serine/threonine protein kinase (Levin and Bartlett-Heubusch 1992) serves to stimulate a Mitogen Activated Protein (MAP) Kinase cascade comprised of Bck1p (Slk1p) (Costigan

et al. 1992, Lee and Levin 1992, Levin *et al.* 1994), Mkk1p/Mkk2p (Irie *et al.* 1993), and Mpk1p (Slt2p) (Lee *et al.* 1993). Mutation of components in the *PKC1-MPK1* pathway have a range of effects, such as; cell lysis, caffeine sensitivity, cell cycle progression defects, and defective cytoskeletal organization. The molecular basis of yeast Pkc1p stimulation is not yet fully understood, however, the GTP-bound form of the small G-protein, Rho1p, has been shown to physically associate with Pkc1p, resulting in Pkc1p activation (Drgonova *et al.* 1996, Kamada *et al.* 1996, Nonaka *et al.* 1995).

Studies of the extracellular matrix of mammalian cells, a structure analogous to the yeast cell wall, have revealed a class of protein receptors known as integrins. Integrins possess a large extracellular domain, a single membrane spanning region and a short cytoplasmic domain. Activation of protein kinases and small GTP-binding proteins such as RhoAp by integrins affects cell adhesion, cellular ion levels, and polarized growth. Recently, Bickle *et al.* (1998) have proposed that disturbances in the cell wall cause activation of the Rho1p GTPase via the Rom2p exchange factor in a manner analogous to integrin signaling. Although the proteome of *Saccharomyces cerevisiae* does not include integrin homologs, there are a number of cell surface proteins topologically resembling integrins that could potentially carry out equivalent extracellular sensing/intracellular signaling processes. These proteins, usually Type I in orientation, contain large extracellular regions rich in serine and threonine residues, single transmembrane domains and relatively small cytoplasmic regions.

WSC1 (*HCS77 /SLG1*) encodes a protein with sensor/signaler-like characteristics that has recently been identified as an upstream activator of Pkc1p (Gray *et al.* 1997, Jacoby *et al.* 1998, Verna *et al.* 1997). Three homologous proteins, encoded by the *WSC2*, *WSC3* and *WSC4* genes, appear to have overlapping activity with Wsc1p since their deletion can increase the severity of *wsc1Δ* phenotypes (Verna *et al.* 1997). *wscΔ* mutants display characteristics of cells with decreased Pkc1p activity, namely reduced growth rate, and sorbitol-suppressible, temperature-dependent cell lysis. Overexpression of genes

presumably downstream of the *WSC* family, such as *RHO1* and *PKC1*, can suppress some of the effects of *WSC* gene deletions. The mechanism by which *WSC* proteins stimulate Pkc1p activity is not yet known, but it has been suggested that Wsc1p might somehow regulate Rho1p activity and thereby affect downstream targets of Rho1p such as Pkc1p (Banuett 1998).

Mid2p, although not a member of the *WSC* family, is another potential extracellular sensor that has been identified as a participant in a number of cellular processes. In wild type *MAT* **a** cells, transcription of *MID2* increases several fold in response to α factor and cells lacking Mid2p die during exposure to α factor (Ono *et al.* 1994). Multicopy *MID2* has been found to suppress a variety of mutant phenotypes including the temperature sensitivity of *mpt5Δ* mutants (Takeuchi *et al.* 1995), growth in profilin (*pfy1Δ*) deficient cells (Marcoux *et al.* 1998), and temperature sensitive growth in *cik1Δ* and *kar3Δ* mutants (Manning *et al.* 1997). Additionally, *KAI1*, an internal fragment of *MID2* was identified as a multicopy inhibitor of excessive protein kinase A (*TPK1*) activity (Daniel 1993).

We identified *MID2* as a high copy activator of the Skn7p transcription factor. A relationship between Mid2p and the cell wall is suggested by a number of genetic interactions between *MID2* and cell wall biosynthesis genes. Alteration of *MID2* gene dosage affects stress-related cell wall chitin deposition, suggesting that *MID2* is partly required for induction of cell wall stress-induced chitin synthesis. Furthermore, genetic interactions between *MID2* and elements of the *PKC1/MPK1* pathway, as well as a requirement for *MID2* during induction of Mpk1p tyrosine phosphorylation under a variety of stress conditions, together suggest a role for Mid2p upstream of the *PKC1-MPK1* cell wall integrity pathway.

3.3 Materials and Methods

Plasmids, Strains and Gene Deletion Constructs

Oligonucleotides used in this study are listed in Table 3.1. Yeast strains used in this study are listed in Table 3.2. Sequences internal to the *KANMX2* and *GFP-HIS3* cassettes are in boldface type. The *MID2* locus, contained within a 2.45 kb *NheI-XhoI* genomic DNA fragment was subcloned into pBS II SK+ at compatible *XbaI* and *SalI* restriction sites. A 2.5 kb *KpnI-SstI* fragment containing *MID2* was excised from this plasmid then inserted into pRS316, pRS425 and pRS426 at corresponding *KpnI-SstI* sites in the polylinker. Deletion of the entire *MID2* open reading frame was accomplished by integration of a *mid2Δ::KANMX2* cassette (Wach *et al.* 1994). *mid2Δ::KANMX2* was generated using the oligonucleotides *mid2Δup* and *mid2Δdown*. Correct insertion of the cassette and deletion of the reading frame was confirmed by PCR analysis of yeast colonies using the oligonucleotides *mid2Δtest* and *KANMX2 internal*. Deletion of *WSC1* was accomplished by replacement of the *WSC1* reading frame with a *wsc1Δ::KANMX2* cassette generated by the oligonucleotides *wsc1Δup* and *wsc1Δ down*. Integration was confirmed using the *KANMX2 internal* test oligonucleotide and the *wsc1Δtest* oligonucleotide. Deletion of *RHO1* was accomplished using a *GFP-HIS3* cassette (Niedenthal *et al.* 1996) and the oligonucleotides *rho1Δup* and *rho1Δdown* to create *rho1Δ::GFP-HIS3*. Correct integration of the deletion cassette into the SEY6210[2] (diploid) strain was confirmed using the oligonucleotides *rho1Δtest* and *GFP-HIS3 internal*. 2:2 segregation of viable:inviable meiotic products was observed after dissection of tetrads derived from heterozygous *rho1::GFP-HIS3 RHO1* diploids. Deletion of *PKC1* was achieved using the oligonucleotides *pkc1Δup* and *pkc1Δdown* to generate the *pkc1Δ::GFP-HIS3* cassette. Integration of the PCR product was confirmed using the oligonucleotides *pkc1Δtest* and *GFP-HIS internal*. Deletion of *MTL1* was accomplished by replacement of the *MTL1*

reading frame with a *mtl1Δ::GFP-HIS3* cassette generated by the nucleotides *mtl1Δup* and *mtl1Δdown*. Correct integration was confirmed using the *GFP-HIS3 internal*, and *mtl1Δtest* nucleotides.

The *RHO1* and *MTL1*genes were amplified from SEY6210 genomic DNA using Expand™ Polymerase (Boehringer Mannheim) and the oligonucleotides *rho1 clone for* and *rho1 clone rev* to generate *RHO1* and *mtl1 clone rev* and either *mtl1 clone for prom.* or *mtl1 clone for start* to generate clones of *MTL1* containing 916 nucleotides of promoter sequence, or a promoter-less clone with only 65 nucleotides 5' of the ATG codon which was used for fusion to the *ADH1* promoter.

Mid2p-HA was created by the insertion of a single copy of the hemagglutinin epitope (YPYDVPDYA) between residues 375 and 376 via site-directed mutagenesis (Kunkel *et al.* 1987) on *MID2* contained in pBSII KS- using the oligonucleotide *HA-insert*. Fidelity of incorporation of the HA epitope was confirmed by DNA sequencing. A *Kpn1-SstI* fragment containing *MID2-HA* was then subcloned into pRS316 and pRS426 at corresponding *KpnI* and *SstI* sites. Mid2p-HA was demonstrated to be functional by the observation that pRS316-*MID2-HA* was fully able to complement a *mid2Δ* mutant in a test for hypersensitivity to α factor (Sigma).

Removal of the serine/threonine-rich region was accomplished by generating an *EcoRI* restriction site by modification of the sequence AAGTTC (nucleotides 641 to 646 in the *MID2* reading frame) to GAATTC via site-directed mutagenesis using the oligonucleotide *EcoRI Insert*. A second *EcoRI* restriction site occurs naturally at positions 102 to 107 in the reading frame. This construct was then digested with *EcoRI*, releasing a 540bp fragment coding for the bulk of the serine/threonine rich region. The remaining plasmid fragment was gel purified (Pharmacia) and re-ligated, creating the continuous in-frame ORF *ΔS/T-MID2*.

Table 3.1 Oligonucleotide Sequences

Oligo Name	Sequence 5'-3'
mid2Δup	GCAGTATCTACTGCACGTTCTTCCGTAAGTAGAGTTAGTT **CTGATATCAAGCTTGCCTCG**
mid2Δdown	TGTTTGGCGTTTGGTAATACGCTATCGCTATCTCTTATTCT **GGTCGACACTGGATGGCGG**
mid2Δtest	GCCTTCAATGAGTTCCAC
KANMX2 internal	**CAACAGGCCAGCCATTAC**
wsc1Δup	ATGAGACCGAACAAAACAAGTCTGCTTCTGGCGTTATTAT **CCGATATCAAGCTTGCCTCG**
wsc1Δ down	TGGATTGACCACTGTTAAAACGTTGTTTTTCCCTCCTGGT **CCGTCGACACTGGATGGCGG**
wsc1Δtest	CGATACAGTAAACTCGAC
HA-insert	GAATTATCACCACGAAATTATTACCCATACGACGTCCCAG ACTACGCTTAATCATATCCATTCATATC
EcoRI Insert	GCAATAAATCCAAGAATTCGGGTCTTTC
KpnI Insert	GGTAACGAATTATCACCACGAAAGGTACCATCATATCCAT TCATATCATTTAG
rho1Δup	ATGTCACAACAAGTTGGTAACAGTATCAGAAGAAAGCTG GTAATC**ATGAGTAAAGGAGAAGAAC**
rho1Δdown	CTATAACAAGACACACTTCTTCTTCTTCTTTTCAGTAGTGT TCTT**GCGCGCCTCGTTCAGAATG**
rho1Δtest	CGACCATCGATCATTCCT
rho1 clone for	TAATGCGGTAGCATTGGACA
rho1 clone rev	AACCTTCCAACAAAACTGAGG
pkc1Δup	ATGAGTTTTTCACAATTGGAGCAGAACATTAAAAAAAAAGA TAGCC**ATGAGTAAAGGAGAAGAAC**
pkc1Δdown	TCATAAATCCAAATCATCTGGCATAAAGGAAAATCCTCTA AACTC**GCGCGCCTCGTTCAGAATG**
pkc1Δtest	CCTGCCAGTGTAATAAGT
GFP-HIS3 internal	**GTATAGTTCATCCATGCC**
mtl1 clone rev	CTTGCCTTCTCCAGAGG
mtl1 clone for prom.	CCACATCAGAGACTTGGG
mtl1 clone for start	GGTTGGTTCGATCTTCGT
mtl1Δup	CGGGTTGGTTCGATCTTCGTTTCCACTTTTAACTTACTCCC **ATGAGTAAAGGAGAAGAAC**
mtl1Δdown	GGGTGATTTTAAGAAGAAAAGTTATGGCAAAGCTGCTTT CG**GCGCGCCTCGTTCAGAATG**
mtl1Δtest	GGTAGAAAAGTGTAGATG

Table 3.2 Strains

Strain	Genotype
L40	*MAT* **a** *his3, trp1, leu2, ade2, LYS2*::(lexAop)$_4$-*HIS3 URA3*::(lexAop)$_8$-*lacZ GAL4*)
TK60	L40/pBTM116-*LEXA-SKN7*/pRS425
TK61	L40/pBTM116-*LEXA-SKN7*/pRS425-*MID2*
SEY6210[2]	*MAT* α/*MAT* **a** *leu2/leu2 ura3/ura3, his3/his3, lys2/lys2, trp1/trp1, suc2/suc2*
SEY6210	*MAT* α *leu2, ura3, his3, lys2, trp1, suc2*
SEY6210a	*MAT* **a** *leu2, ura3, his3, lys2, trp1, suc2*
TK82	SEY6210a/pRS426
TK83	SEY6210a/pRS426-*MID2*
TK84	SEY6210a/pRS426-*MID2-HA*
TK85	SEY6210a/pRS426-*ΔS/T-MID2-HA*
TK86	SEY6210a/pVT101U
TK87	SEY6210a/pVT101U-*MID2*
TK88	SEY6210a *mid2Δ::KANMX2*
TK89	SEY6210a *mid2Δ::KANMX2*/pRS425
TK90	SEY6210a *mid2Δ::KANMX2*/pRS425-*WSC1*
TK91	SEY6210a *mid2Δ::KANMX2*/pRS425-*WSC2*
TK92	SEY6210a *mid2Δ::KANMX2*/pRS425-*RHO1*
TK93	SEY6210a *mid2Δ::KANMX2*/pFL44
TK94	SEY6210a *mid2Δ::KANMX2*/pFL44-*MPK1-HA*
TK95	SEY6210a/pRS425
TK96	SEY6210a/pFL44
TK97	SEY6210a/pFL44-*MPK1-HA*
TK98	SEY6210a/pRS426-*MID2-GFP*
TK99	SEY6210a *chs3Δ::LEU2*/pVT101U
TK100	SEY6210a *chs3Δ::LEU2*/pVT101U-*MID2*
TK101	SEY6210[2] *kre6Δ::HIS3/KRE6 mid2Δ::KANMX2/MID2*
TK102	SEY6210[2] *kre9Δ::HIS3/KRE9 mid2Δ::KANMX2/MID2*
TK103	SEY6210[2] *fks1Δ::HIS3/FKS1 mid2Δ::KANMX2/MID2*
TK104	SEY6210[2] *wsc1Δ::KANMX2 mid2Δ::KANMX2/MID2*
TK105	SEY6210[2] *mtl1Δ::GFP-HIS3 mid2Δ::KANMX2/MID2*

Westerns and Membrane Association Tests

For membrane association tests, total cell extracts were prepared from mid log phase cultures grown in selective medium by vigorous vortexing in lysis buffer (50mM Tris 7.5, 1mM EDTA, 5% glycerol) in the presence of glass beads and protease inhibitors (Complete™ protease inhibitor cocktail, Boehringer Mannheim). The resulting slurry was centrifuged at 2500g at 4°C for 5 min. to remove cell walls and unbroken cells. The resulting supernatant was then divided and the individual aliquots were subjected to either centrifugation at 15000g at 4°C for 30 min. or treatment with sodium chloride or sodium carbonate or urea or Triton X-100 then subjected to centrifugation at 60000g at 4°C for 30 min. Post centrifugation, supernatants were withdrawn, and pellets were resuspended in a volume of lysis buffer equal to the supernatant. Samples were resolved by SDS-PAGE then subjected to Western blotting. Immunodetection of Mid2p-HA was achieved using anti-HA monoclonal antibody 12CA5 (Babco) at 1:1000 dilution and HRP-conjugated anti-mouse secondary antibody (Amersham Life Sciences) at a 1:1000. Bands were visualized using ECL (Amersham Life Sciences). For other SDS-PAGE and Western blotting procedures, total cell lysates were prepared using lysis buffer consisting of 2% Triton-X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA.

Localization of Mid2p

A *MID2-GFP* fusion was generated by modifying the coding sequence of *MID2* (contained in pBSII KS-) immediately upstream of the TAA stop codon (TTATTA) via site-directed mutagenesis to a *KpnI* restriction site (GGTACC) using the oligonucleotide *Kpn1 Insert*. Clones positive for the incorporation of the *KpnI* site were then confirmed by sequencing. Creation of an in-frame fusion of *MID2* to *GFP* (F64L S65T kindly provided by U. Stochaj) was accomplished by a three way ligation involving pRS426 or pRS316

(with *Xho1/EcoRI* ends) *MID2* (with *Xho1/Kpn1* ends) and *GFP* (with *Kpn1/EcoRI* ends). Correct orientation of the ligation products was confirmed by diagnostic restriction digests. Function of the *MID2-GFP* fusion was demonstrated by observation that pRS316-*MID2-GFP* fully complements *mid2Δ* mutants for α factor hypersensitivity. Localization of Mid2p-GFP was accomplished by examination of live, mid-log phase *mid2Δ* cells carrying pRS316-*MID2-GFP* and pRS426-*MID2-GFP*.

Calcofluor White Sensitivity Tests

To test strains for sensitivity to calcofluor white, mid log phase cells were diluted, then spotted onto either rich YEPD agar plates containing the indicated amount of calcofluor white, or onto selective medium buffered with 10g/L MES and adjusted to pH 6.2. Plates were incubated for 48-72 hours at 30°C in a dark environment.

Multicopy Suppression of α Factor-Induced Death

Cultures of cells containing either control plasmids or plasmids bearing genes of interest were diluted to $3x10^4$ cells/ml in buffered (85mM succinic acid, 19mM sodium hydroxide) YNB liquid medium containing appropriate amino acids. Triplicate aliquots of each strain were removed and spread on selective solid medium. α factor was then added to liquid cultures to a final concentration of 1μM, and these cultures were incubated at 30°C for 330 minutes before a second series of aliquots were removed and plated. Petri dishes were incubated for 48 hours at 30° C, resulting in the formation of colonies derived from single cells. The number of colonies on each petri dish was counted, and survival of α factor exposure was measured by the difference between the number of colonies on pre and post-α factor petri dishes for each strain.

Chitin Assays

Total cellular chitin was measured essentially as described by Bulawa *et al.* (1986) and outlined here (personal communication C. Bulawa). Washed cells (~50mg wet weight) were suspended in 500ul of 6% KOH and incubated at 80°C for 90 min. After cooling to room temperature, 50ul of glacial acetic acid was added. Insoluble material was washed twice with water, and resuspended in 250ul of 50mM sodium phosphate pH 6.3. 2mg of *Streptomyces griseus* chitinase (Sigma) was added, and tubes were incubated at 25°C with gentle agitation for 2 hours. Tubes were centrifuged at 15000g for 5 minutes at room temperature, and 250ul of supernatant was transferred to a fresh tube to which 1mg of *Helix pomatia* β-glucuronidase (Sigma) was added. Tubes were incubated at 37°C for 2 hours with gentle agitation and then assayed for *N*-acetylglucosamine content.

Measurement of Mpk1p-HA Phosphotyrosine Content

Mid-log phase cultures of cells expressing Mpk1p-HA or a vector only control were exposed to calcofluor white (40μg/ml for 30 min.), α factor (4μM for 3 hours) or high temperature (37°C for 3 hours). 1ml of culture was centrifuged (15000g 30sec. room temperature), the supernatant was aspirated, and the cell pellet was resuspended in 50ul of 1X SDS loading buffer and boiled for 4 min. Cell debris was pelleted by centrifugation, and 10ul of supernatant per sample was subjected to SDS-PAGE and Western blotting. Tyrosine phosphorylation of proteins was visualized by incubation of blots with the phosphotyrosine-specific antibody 4G10 (Gift of J. Lee) at 1:3300 dilution, and anti-mouse HRP-conjugated secondary antibody at 1:2000. Blots were then stripped and reprobed using the anti-HA monoclonal antibody 12CA5 to verify equal loading of Mpk1p-HA in each lane.

3.4 Results

MID2 Stimulates Skn7p Transcriptional Activity

Skn7p, a transcription factor containing a region of homology to bacterial two-component response-regulator proteins was isolated by Brown *et al.* (1993) as a high copy suppressor of growth defects in *kre9Δ* mutants. A screen was performed to identify genes which, when overexpressed, would stimulate the Skn7p-LexA dependent transcription of a $(lexA_{op})_4$-His3 reporter (Pagé *et al.* 1996). In this procedure, the L40 reporter strain carrying Skn7p-LexA was transformed with a Yep13-based multicopy genomic bank. Plasmid clones were extracted from colonies which could grow on synthetic media lacking histidine and including 10 mM 3-Amino-1,2,4-Triazole (3AT) to squelch His3p activity resulting from basal transcription of *HIS3*.

Ten groups of Activator of *SKN7* (*ASK*) clones were identified; *ASK1-ASK10*. Sequence analysis revealed that *ASK5*, *ASK7*, and *ASK9* contained a common gene; *YLR332W* (*MID2*). We subcloned the *MID2* gene including 627 nucleotides 5' of the codon for the start methionine, and 737 nucleotides 3' of the stop codon into pRS425 and directly tested the ability of *MID2* to stimulate Skn7p-LexA transcriptional activity. Multicopy *MID2* is able to strongly induce Skn7p-LexA activity compared to a vector only control, permitting reporter strain growth on media lacking histidine and containing 30mM 3AT (Fig. 3.1). This effect is dependent on an interaction between *MID2* and *LEXA-SKN7* since overexpression of *MID2* alone does not activate transcription of the reporter (not shown). In addition, we also measured the induction of ß-galactosidase activity from the $(lexA_{op})_8$-lacZ locus and found that multicopy *MID2* was able to induce a seven to ten fold, Skn7-LexA-dependent, increase in β-galactosidase activity (not shown).

Figure 3.1 Multicopy *MID2* activates Skn7p-LexA-dependent transcription of *HIS3* which allows growth on medium lacking histidine. Reporter strains containing Skn7p-LexA and either pRS425 (TK60) or pRS425-*MID2* (TK61) on selective medium lacking histidine, and containing 30mM 3AT.


Skn7p-LexA
+pRS425
Skn7p-LexA
+pRS425-MID2

Characterization and Subcellular Localization of Mid2p

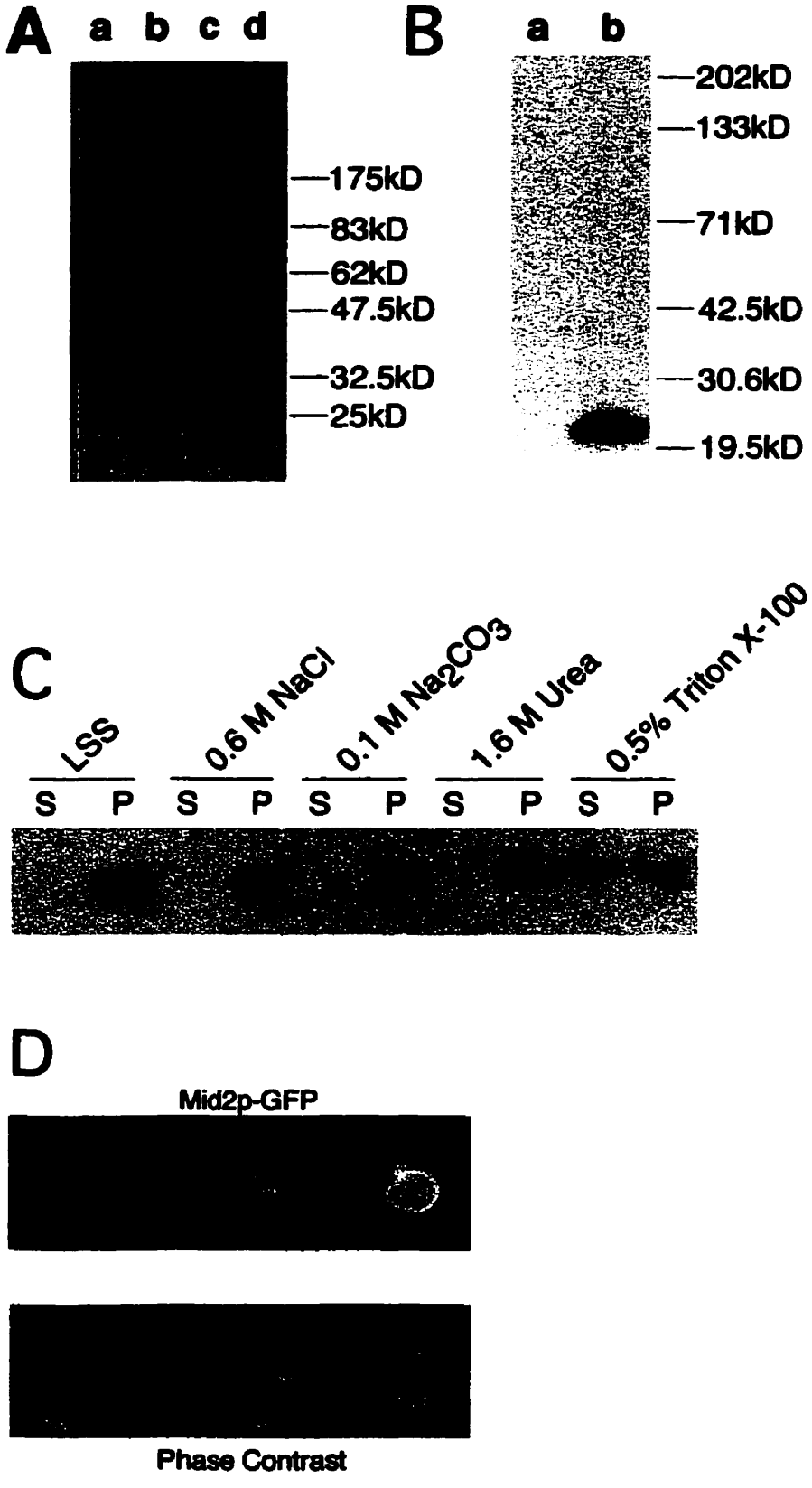
MID2 is predicted to encode a Type I, membrane-spanning protein, containing an N-terminal secretion signal sequence followed by a domain of approximately 200 amino acids consisting of ~62% serine and threonine residues. The C-terminal third of the protein contains a single predicted transmembrane domain and a short charged domain rich in aspartic acid residues, suggested to resemble a calcium binding domain (Ono *et al.* 1994). Although Mid2p has overall structural similarity to members of the *WSC* family, there are two important differences. Firstly, Mid2p does not contain an extracellular cysteine-rich motif that is characteristic of the *WSC* proteins. Secondly, apart from the repetitive serine/threonine region, there is no statistically significant amino acid residue sequence similarity between Mid2p and Wsc proteins.

To examine physical characteristics of Mid2p, a functional hemagglutinin-tagged protein (Mid2p-HA) was generated. After proteolytic processing of the signal peptide, Mid2p-HA is predicted to have a molecular weight of approximately 39kD. However, Mid2p-HA migrates with an apparent molecular weight of approximately 200kD on SDS-PAGE (Fig. 3.2A lane b). The predicted Type I orientation of Mid2p suggests that if Mid2p were to be plasma membrane localized, the serine/threonine-rich region would reside on the exterior face of the cell. Since serine/threonine rich regions of extracellular protein domains can receive O-linked mannosylation as the protein travels through the secretory pathway (Tanner and Lehle 1987), we examined Mid2p for evidence of this modification. When isolated from *pmt1Δ pmt2Δ* mutants (deficient in O-linked mannosylation (Lussier *et al* 1995)), Mid2p-HA migrates on SDS-PAGE close to the predicted size of 39 kD (Fig. 3.2A lane d), displaying a shift of roughly 160kD compared to Mid2p-HA expressed in wild type cells. To verify that it was the serine/threonine domain that was the recipient of the O-mannosylation, we excised this region from Mid2p-HA, generating ΔS/T Mid2p-HA. On SDS-PAGE, ΔS/T Mid2p-HA also migrates near its predicted molecular weight of

23kD (Fig. 3.2B), strongly suggesting that the serine/threonine-rich region is the recipient site of O-linked mannosylation. ΔS/T Mid2p-HA localizes to the same subcellular location as Mid2p-HA (not shown, see end of this section), however pRS316-ΔS/T Mid2p-HA is unable to complement a *mid2Δ* mutant for sensitivity to α factor, indicating that Mid2p requires the serine-threonine rich domain for activity. Together, these results suggest that extensive, functionally important modification of Mid2p occurs on the extracellular serine/threonine-rich region.

To verify the prediction that Mid2p is an integral membrane protein, partially purified extracts from cells expressing Mid2p-HA were fractionated into supernatant (soluble) and pellet (membrane containing) portions by centrifugation. Mid2p-HA is found exclusively in the LSS (Low Speed Spin, 15000g) pellet fraction, implying membrane association (Fig. 3.2C). To test whether this membrane association was peripheral or integral, partially purified, membrane-containing cell extracts were treated prior to ultracentrifugation with sodium chloride, sodium carbonate, or urea to disrupt peripheral interactions, or with Triton X-100 to disrupt integral membrane association. Only Triton X-100 was capable of solubilizing a significant proportion of Mid2p-HA (Fig. 3.2C), strongly suggesting that Mid2p-HA is an integral membrane protein.

Figure 3.2 Cell Biology of Mid2p. **(A)** Immunoblot analysis of cell extracts from **a)** TK82 (vector only) **b)** TK84 (*MID2-HA*) **c)** *pmt1Δ pmt2Δ* (vector only) **d)** *pmt1Δ pmt2Δ* (*MID2-HA*) **(B)** Immunoblot analysis of cell extracts from **a)** TK82 (vector only) **b)** TK85 (*ΔS/T-Mid2p-HA*) **(C)** Immunoblot analysis of cell fractions from TK84 to demonstrate membrane association of Mid2p **(D)** In cells expressing pRS426-*MID2-GFP* (TK98), Mid2p-GFP is localized to the cell periphery.


A
a b c d
175kD
83kD
62kD
47.5kD
32.5kD
25kD
B
a b
202kD
133kD
71kD
42.5kD
30.6kD
19.5kD
C
LSS
0.6 M NaCl
0.1 M Na2CO3
1.6 M Urea
0.5% Triton X-100
S P S P S P S P S P
D
Mid2p-GFP
Phase Contrast

There does not appear to be a fraction of Mid2p-HA covalently associated with the cell wall since treatment of purified cell wall fractions with β-1,3-glucanase (either laminarinase or Quantazyme™) before solubilization of proteins by treatment with SDS does not release any detectable Mid2p-HA (not shown).

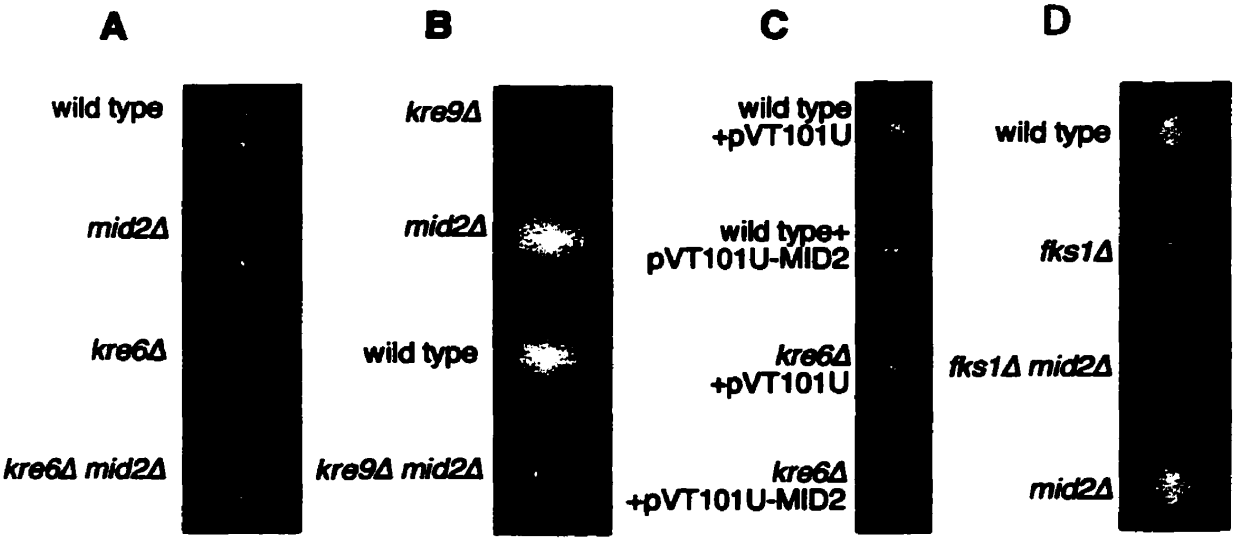
Direct immunofluorescence microscopy was performed to establish the subcellular localization of Mid2p. A functional Mid2p-GFP fusion protein was constructed by inserting *GFP* immediately upstream of the *MID2* stop codon. Examination of fluorescing cells maintaining either centromeric (not shown) or multicopy Mid2p-GFP (Fig. 3.2D) reveals Mid2p-GFP distribution to be largely confined to the periphery of cells, consistent with a plasma membrane localization. Expression of native GFP alone produces a diffuse fluorescence pattern throughout the cell (not shown). Indirect immunofluorescence of cells expressing Mid2p-HA revealed an identical pattern of staining (not shown). No polarized localization of Mid2p-GFP to specific regions of the surface such as the bud tip, or bud neck was detected, however approximately 20% of α factor-treated cells (n=100) display faint preferential staining at the sub-apical region of the mating projection (not shown).

Interactions between MID2 and Cell Wall Biosynthesis Genes

Since the O-mannosylated, extracellular domain of Mid2p is predicted to be oriented towards the cell wall, we explored a possible relationship between Mid2p and the cell wall, by searching for genetic interactions between *MID2* and genes known to be involved in cell wall construction. Deletion of *MID2* in two viable but slow growing β-1,6-glucan synthesis mutants, *kre6Δ* (Roemer and Bussey 1991) and *kre9Δ* (Brown and Bussey 1993), partially restores growth rate. (Fig. 3.3A, 3.3B). This effect is not due to differential timing of spore germination since increased growth rate in the absence of *MID2* (~20% reduction in doubling time) is observed for *kre6Δ* cells cultured in liquid medium. Also, re-introduction of *MID2* on a centromeric plasmid causes *kre6Δ mid2Δ* cells and *kre9Δ mid2Δ* cells to

resume a slower growth rate (not shown). Interestingly, high copy expression of *MID2* in the *kre6Δ* mutant has a strong negative effect on growth rate, (Fig. 3.3C). Optical density measurement of cell growth in liquid culture revealed that high copy expression of *MID2* (pRS426-*MID2*) in *kre6Δ* cells resulted in a ~300% increase in doubling time compared to a vector-only control. This reduction in growth speed is much more pronounced in *kre6Δ* mutants than in wild type cells, where doubling time increases by only 15% over a vector-only control for cells carrying pRS426-*MID2*. Interestingly, high copy expression of *MID2* in the growth deficient *pmt1Δ pmt2Δ* mutants, where Mid2p is under mannosylated, also results in a reduction in growth rate. This suggests that although the serine/threonine-rich domain itself is indispensable for Mid2p activity, extensive O-linked mannosylation of this region may not be essential for Mid2p function.

Figure 3.3 Deletion of *MID2* has effects on growth of different cell wall mutants. (**A**) Representative tetra-type tetrad from TK101 (*kre6Δ mid2Δ* heterozygous diploid). (**B**) Representative tetra-type tetrad from TK102 (*kre9Δ mid2Δ* heterozygous diploid). (**C**) Single cells containing the indicated plasmids were placed on selective agar and grown at 30°C for four days. Photo is representative of effect seen in 3 isolates each of 3 transformations. (**D**) Representative tetra-type tetrad from TK103 (*fks1Δ mid2Δ* heterozygous diploid).


A
wild type
mid2Δ
kre6Δ
kre6Δ mid2Δ
B
kre9Δ
mid2Δ
wild type
kre9Δ mid2Δ
C
wild type +pVT101U
wild type+ pVT101U-MID2
kre6Δ +pVT101U
kre6Δ +pVT101U-MID2
D
wild type
fks1Δ
fks1Δ mid2Δ
mid2Δ

Examination of cell wall glucan content revealed that wild type and *mid2Δ* cells have no differences in β-1,6 and β-1,3-glucan content. Similarly, *kre6Δ* and *kre6Δ mid2Δ* mutants have comparable levels of both glucan species (not shown), suggesting, at least for the *kre6Δ* mutant, that increased growth rate induced by loss of *MID2* is not caused by a restoration of β-1,6-glucan synthesis. To determine if *MID2* genetically interacts with the β-1,3-glucan synthesis pathway, we examined the consequence of loss of *MID2* in *fks1Δ* mutants (Douglas *et al.* 1994, Eng *et al.* 1994, Ram *et al.* 1995). In contrast to the restorative effect that deletion of *MID2* had in ß-1,6-glucan mutants, loss of MID2 in *fks1Δ* cells causes inviability (Fig 3.3D). This phenotype is not reversible by the addition of 1M sorbitol or 30mM calcium chloride (not shown). Overexpression of *MID2* in an *fks1Δ* mutant does not result in an inhibition of growth like that seen in *kre6Δ* mutants with high copy *MID2* (not shown).

MID2 Affects Chitin Synthesis Under Stress Conditions

Although chitin comprises a small percentage of the cell wall weight, its contribution to wall integrity is vital. Calcofluor white is a fluorescent dye that intercalates into nascent chitin chains, preventing microfibril assembly (Elorza *et al.* 1983). At sufficient concentrations, calcofluor white can kill cells through interference with cell wall assembly. *mid2Δ* cells display significant resistance to calcofluor white. At a concentration of 20μg/ml calcofluor white on rich medium, wild type cells are killed while *mid2Δ* cells can grow without apparent inhibition (Fig 3.4A).

Figure 3.4 Dosage of *MID2* affects sensitivity to calcofluor white. Mid log phase cells were diluted to a concentration of $3x10^6$cells/ml. 5ul of this suspension and three subsequent 10 fold serial dilutions were each spotted onto the indicated medium. **(A) a)** SEY6210**a** (wild type) and **b)** TK88 (*mid2Δ*) cells were spotted onto YEPD containing 0 and 20μg/ml calcofluor white. **(B) a)** TK82 (wild type+pRS426), **b)** TK83 (wild type+pRS426-*MID2*), **c)** TK86 (wild type+pVT101U), and **d)** TK87 (wild type+pVT101U-*MID2*) were spotted on uracil dropout medium containing 0 and 2.5μg/ml calcofluor white. **(C) a)** TK86 **b)** TK87, **c)** TK99 (*chs3Δ*+pVT101U) and **d)** TK100 (*chs3Δ*+pVT101U-*MID2*)were spotted onto uracil dropout medium containing 0, 2.5 and 15μg/ml calcofluor white.

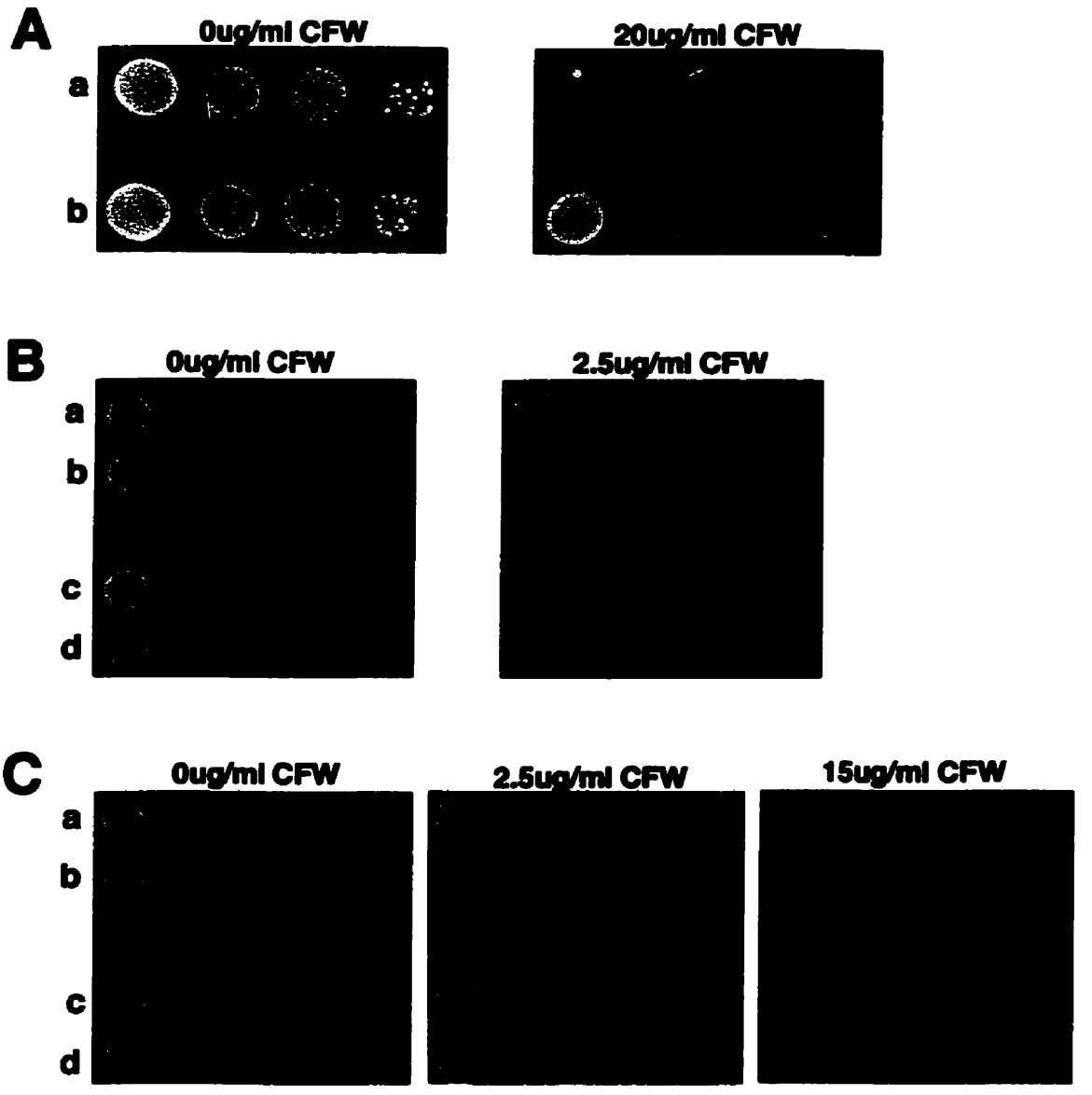

A
0ug/ml CFW
20ug/ml CFW
a
b
B
0ug/ml CFW
2.5ug/ml CFW
a
b
c
d
C
0ug/ml CFW
2.5ug/ml CFW
15ug/ml CFW
a
b
c
d

Table 3.3 Measurement of total cellular chitin

Condition	Strain	nMoles GlcNAc/mg cell weight	Increase from Control (nMoles GlcNAc/mg wet cell weight)	*MID2*-Dependent Change
A) YEPD (Control)	Wild Type *mid2Δ*	3.54 (+/-0.06) 3.60 (+/-0.31)	- -	
B) YEPD+ Calcofluor White 10μg/ml 2 hrs.	Wild Type *mid2Δ*	8.96 (+/-0.16) 6.82 (+/-0.14)	5.42 3.22	-40%
C) YEPD	*kre6Δ* *kre6Δ mid2Δ*	9.50 (+/-0.43) 7.90 (+/-0.08)	5.96 (from wild type) 4.30 (from *mid2Δ*)	-28%
D) YEPD 4μM alpha factor 3 hrs.	Wild Type *mid2Δ*	5.05 (+/-0.19) 3.91 (+/-0.05)	1.51 0.31	-79%
E) YNB (uracil dropout)	Wild Type + pVT101U Wild Type + pVT101U-*MID2*	4.65 (+/-0.65) 11.87 (+/-0.22)	- -	+255%

Since resistance to calcofluor white is a phenotype often associated with defects in chitin synthesis, one possibility is that calcofluor white resistance of *mid2Δ* cells is a consequence of reduced chitin synthesis. Measurement of chitin levels in wild type and *mid2Δ* cells revealed that they have identical chitin contents when grown in rich medium, suggesting that Mid2p is not required for basal chitin production under such optimum growth conditions (Table 3.3 Row A). Because exposure to calcofluor increases chitin production *in vivo* (Roncero and Duran 1985), we tested whether *MID2* is required for synthesis of supplemental chitin by measuring the chitin content of cells that had been challenged with sub-lethal concentrations of calcofluor white. We then calculated the amount of new chitin synthesized in response to calcofluor white challenge by subtracting the amount of chitin produced under non-stressed conditions from the total chitin measured after calcofluor white exposure.

Interestingly, *mid2Δ* mutants contained 40% less new, stress-induced, total cell wall chitin than wild type cells when cultures were grown in the presence of 10μg/ml calcofluor white for two hours prior to harvesting (Table 3.3 Row B). This attenuation of calcofluor white-induced chitin synthesis is likely to be at least part of the cause of calcofluor white resistance in *mid2Δ* cells. To determine if Mid2p might be required for supplementary chitin synthesis under a broader range of cell wall stresses, we looked for Mid2p-dependent changes in cellular chitin content in two other circumstances. It has been observed that cell wall mutants typically have higher cell wall chitin levels than wild type cells (Popolo *et al.* 1997, Ram *et al.* 1998). Analysis of chitin content revealed that *kre6Δ* cells have a greater than 2.5 fold increase in total chitin over wild type cells. Similar to the effect seen in calcofluor white-challenged cells, loss of *MID2* causes a small but significant decrease (~28%) in extent of stress-induced chitin synthesis in *kre6Δ* mutants (Table 3.3 Row C).

Another situation known to increase chitin production is shmoo formation in response to mating pheromone (Schekman and Brawley 1979). After induction of

projection formation by α factor, *MAT* **a** *mid2Δ* mutants had synthesized almost 80% less new chitin than wild type cells (Table 3.3 Row D). These observations suggest that Mid2p is partially required for production of supplementary wall chitin under conditions of wall damage (by mutation or calcofluor white) or morphological change (shmoo formation).

To further address the relationship between *MID2* and chitin synthesis, growth of cells overexpressing *MID2* was examined on calcofluor white-containing medium. Cells habouring *MID2* on a 2μ plasmid have reduced viability at a concentration of 2.5μg/ml calcofluor white, and cells expressing *MID2* from the strong constitutive *ADH1* promoter at 2μ levels are completely inviable on 2.5μg/ml calcofluor white (Fig. 3.4B). Since calcofluor white resistance in *mid2Δ* cells is associated with reduced supplementary chitin synthesis, this finding suggests that over expression of *MID2* might cause an increase in wall chitin content, conferring hypersensitivity to this drug. Indeed, analysis of total chitin revealed that cells carrying *ADH1* promoter-driven *MID2* had approximately 250% more chitin than cells without multicopy *MID2* (Table 3.3 Row E).

Because Chs3p is responsible for the bulk of lateral wall and bud scar chitin, and deletion of *CHS3* leads to a 10 fold reduction in total cellular chitin content, and strong resistance to calcofluor white, we next tested whether the hypersensitivity to calcofluor white caused by *MID2*-overexpression is mediated through Chs3p activity. Spot-testing of *chs3Δ* cells containing multicopy *MID2*, with either its own or the *ADH1* promoter on calcofluor white-containing medium revealed that *CHS3* is required for high copy *MID2* to confer calcofluor white hypersensitivity (Fig. 3.4C). This finding suggests that Mid2p might ultimately affect extra chitin synthesis through some form of regulation of Chs3p activity.

MID2 Interacts with the Cell Integrity Pathway

To identify additional *MID2* interactors, we performed a screen for multicopy suppressors of the *mid2Δ fks1Δ* synthetic lethality (plasmids were selected for their ability

to promote growth in *mid2Δ fks1Δ* +pRS316-*FKS1* cells after loss of pRS316-*FKS1* was promoted by replication onto medium containing 5-Fluoroorotic acid). Two of the genes identified as *mid2Δ fks1Δ* suppressors were *PKC1* and *WSC1*. Several genetic interactions between *MID2* and *WSC1* suggest that these genes possess overlapping activities. First, *mid2Δ wsc1Δ* cells are inviable at 22°C or at 30°C on YEPD medium. Inclusion of sorbitol (between 0.3 and 1M) in medium permits partial restoration of growth, suggesting that *mid2Δ wsc1Δ*, like *pkc1Δ* mutants are prone to lysis without osmotic support (Fig. 3.5). When transferred from osmotically supported medium to YEPD, *mid2Δ wsc1Δ* mutants arrest growth with a small bud. After 36 hours on YEPD, approximately 85% of *mid2Δ wsc1Δ* cells have small buds, while between 6% and 8% of wild type, *mid2Δ*, and *wsc1Δ* cells display a small bud (greater than 150 cells counted per genotype) (Fig. 3.6). Further suggesting a functional relationship between *MID2* and *WSC1*, high copy expression of *MID2* partially suppresses the growth defect of *wsc1Δ* mutants (not shown). Finally, overexpression of *WSC1* is able to relieve the sensitivity of *mid2Δ* cells to α factor (Fig. 3.7). Together, these findings indicate a functional overlap between Mid2p and Wsc1p.

Since Wsc1p and its homologs have been implicated in Pkc1p activation, and the *mid2Δ wsc1Δ* small-budded terminal phenotype resembles that of cells lacking Pkc1p activity (Levin and Bartlett-Heubusch 1992), we examined the possibility that Mid2p may also contribute to regulation of the *PKC1-MPK1* pathway. Like *pkc1Δ* mutants, *mid2Δ pkc1Δ* cells are inviable on medium without osmotic support, however, these double mutants are able to grow at a rate similar to *pkc1Δ* mutants on medium containing 1M sorbitol at both 22°C and 30°C (not shown). Moreover, multicopy *MID2* does not alter the growth rate of *pkc1Δ* cells (not shown), suggesting that genetically, *PKC1* acts downstream of *MID2*.

Figure 3.5 Genetic interaction between *MID2* and *WSC1*. Representative tetra-type tetrads of TK104 (*mid2Δ wsc1Δ* heterozygous diploid) dissected onto either YEPD or YEPD plus 1M sorbitol. YEPD Plates were incubated for 60 hours at 30°C, YEPD 1M sorbitol plates were incubated for 80 hours at 30°C.

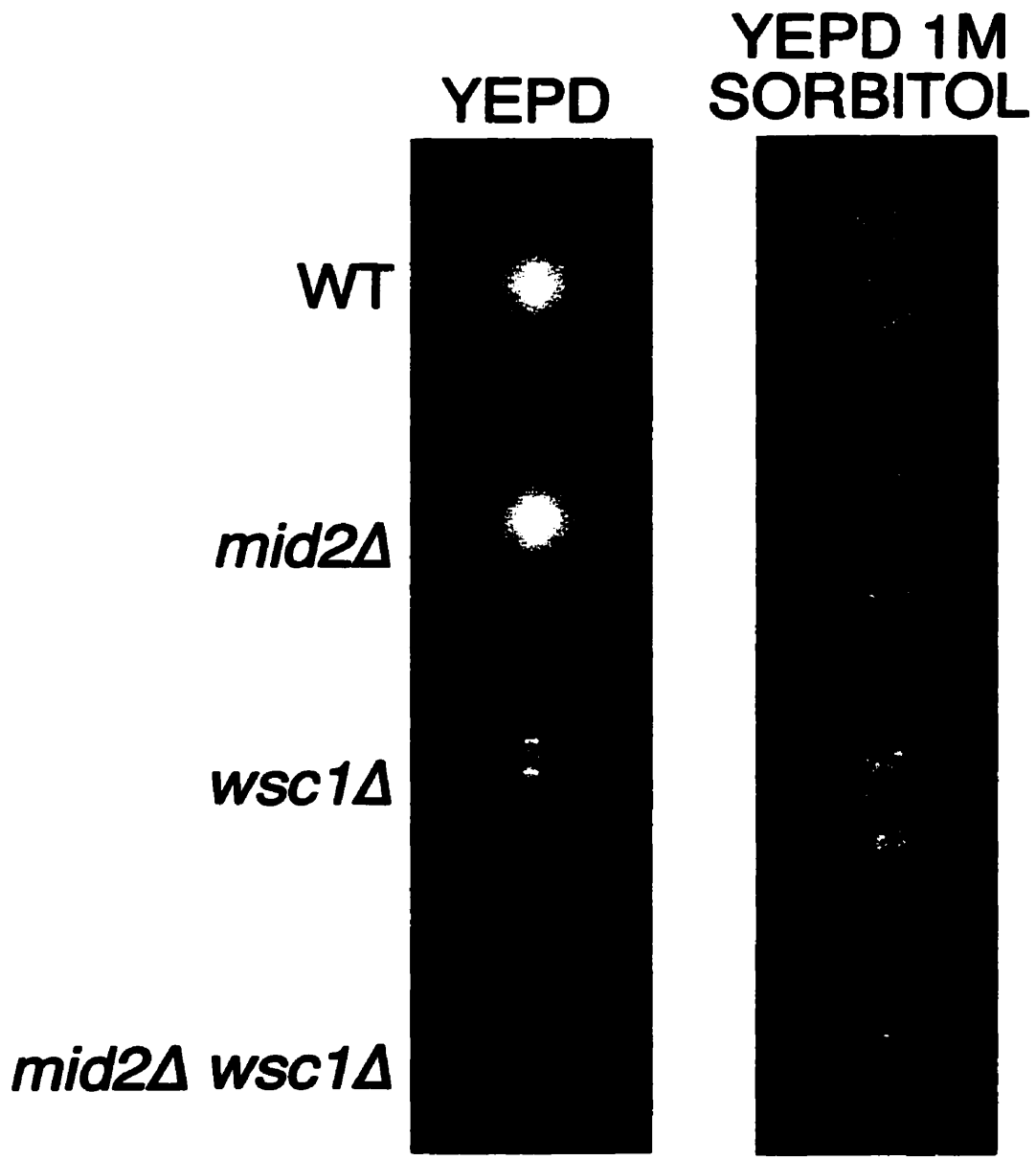

YEPD
YEPD 1M SORBITOL
WT
mid2Δ
wsc1Δ
mid2Δ wsc1Δ

Figure 3.6 Terminal Phenotype of *mid2Δ wsc1Δ* mutants. Wild type, *mid2Δ*, *wsc1Δ*, and *mid2Δ wsc1Δ* cells grown in liquid YEPD medium + 0.3M sorbitol were pelleted, washed once with medium, then resuspended in YEPD medium without sorbitol and incubated at 30°C for 8 hours. Cells were then fixed with 3.7% formaldehyde and photographed.

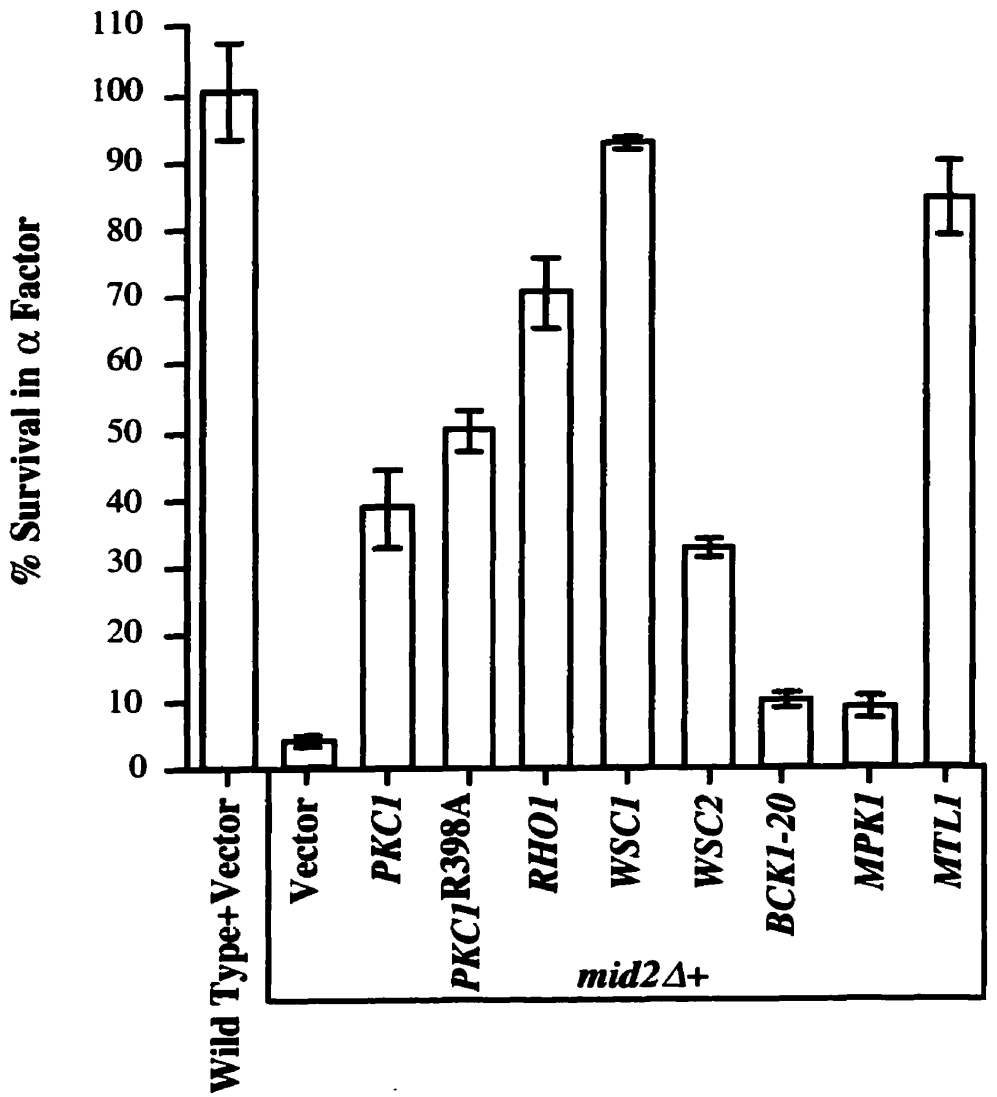
Wild Type

mid2Δ

wsc1

mid2Δ wsc1Δ

Figure 3.7 Members of the cell integrity pathway suppress α factor-induced death in *mid2Δ* mutants. Wild type cells containing pRS426 vector only and *mid2Δ* mutants carrying pRS426, YEP13-*PKC1*, pBM743-*PKC1*^{R398A} (Gal-driven, hyperactive *PKC1*), pRS426-*RHO1*, pRS426-*WSC1*, pRS426-*WSC2*, pRS316-*BCK1-20* (hyperactive *BCK1*), YEP352-*MPK1*, pRS425-*MTL1* in liquid medium were exposed to α factor. Percentage of survival was measured by spreading liquid medium pre and post α factor exposure (330 min.) on petri dishes and counting colonies derived from single cells.


% Survival in α Factor
110
100
90
80
70
60
50
40
30
20
10
0
Wild Type+Vector
Vector
PKC1
PKC1R398A
RHO1
WSC1
WSC2
BCK1-20
MPK1
MTL1
mid2Δ+

To further explore the potential role of Mid2p in *PKC1-MPK1* pathway regulation, we examined whether Mid2p influences Mpk1p activation. The Mpk1p MAP kinase is activated through tyrosine phosphorylation during mating projection formation (Bueher *et al.* 1997, Zarzov *et al.* 1996). We found that compared to wild type cells, *mid2Δ* mutants have substantially less tyrosine phosphorylation on Mpk1p-HA after exposure of cells to α factor (Fig. 3.8A). This observation suggests that under-activation of the cell integrity pathway might be, at least in part, responsible for pheromone-induced death in *mid2Δ* mutants.

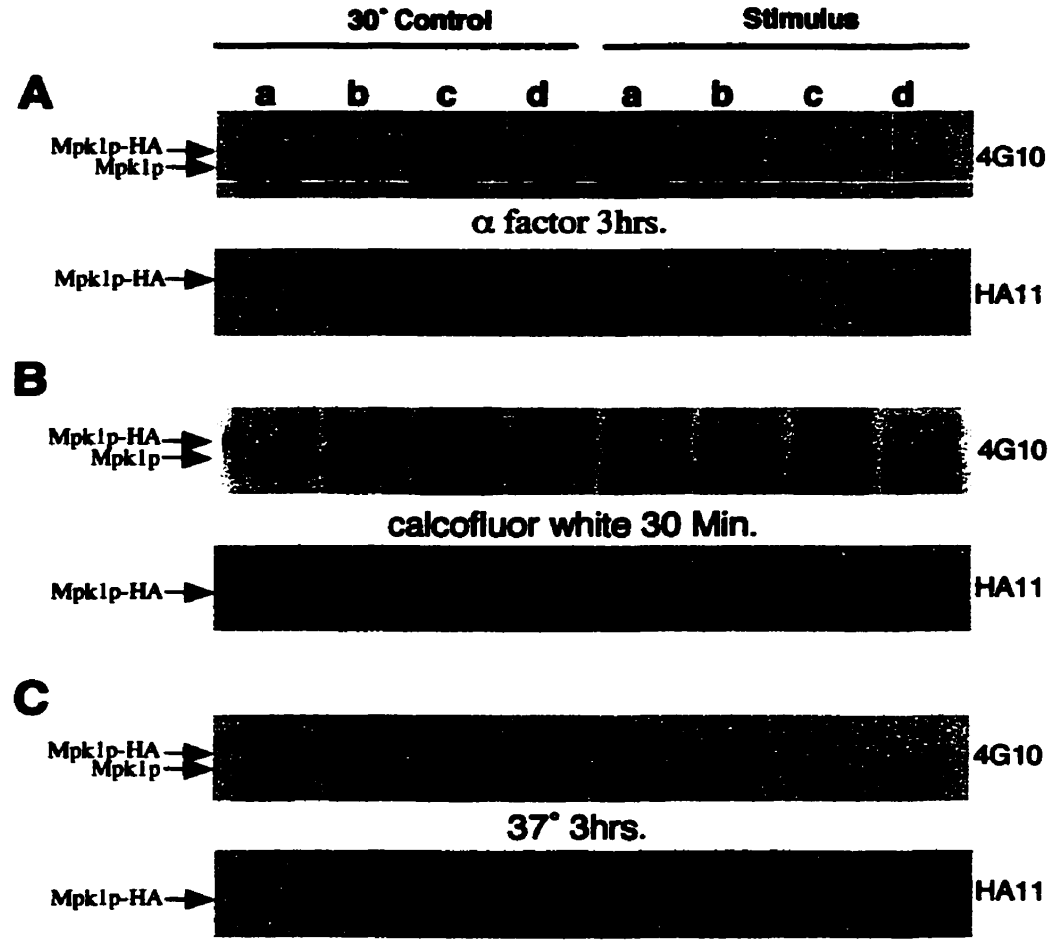
The finding that overexpression of *PKC1*, *PKC1*^{R398A} (a hyperactive allele), *RHO1*, *WSC1*, and *WSC2* can suppress α factor-induced death in *mid2Δ* mutants (Fig. 3.7) independently supports this conclusion. However, expression of either *bck1-20* (a hyperactive allele of *BCK1*), or *MPK1* does not suppress this phenotype, and at high concentrations of pheromone (>4 μM) only some of the most upstream elements of the cell integrity pathway including *RHO1*, *WSC1*, and *WSC2* can prevent mating pheromone-induced death (not shown).

To explore the genetic relationship between *MID2* and *RHO1*, we tested whether *MID2* overexpression suppresses *rho1Δ* mutants. Because cells lacking Rho1p are inviable, even at low temperature on medium with osmotic support, *rho1Δ* heterozygous diploids were transformed with high copy *MID2* then sporulated, and the resulting asci were dissected. No viable *rho1Δ* mutants were recovered (36 tetrads analyzed), suggesting that high copy *MID2* is unable to suppress the lack of Rho1p. Since overexpression of *RHO1* can suppress α factor-induced death in *mid2Δ* mutants, but high copy *MID2* cannot suppress inviability of *rho1Δ* mutants, it appears that Mid2p might act upstream of, or in parallel with, Rho1p.

Interestingly, Mpk1p-HA tyrosine phosphorylation is also increased in wild type cells in response to exposure to calcofluor white. This effect is dependent on the presence of Mid2p, since there is a deficit of this phosphorylation on Mpk1-HA in *mid2Δ* cells (Fig.

3.8B). *MID2*-dependent increase in tyrosine phosphorylation of native Mpk1p in response to calcofluor white and mating pheromone is not readily apparent in Fig. 3.8A and 3.8B, perhaps due to lower relative abundance of native Mpk1p versus (2μ-borne) Mpk1-HA. Also, for the chosen time points, a lower extent of tyrosinse phosphorylation on Mpk1-HA is seen for calcofluor white and mating factor exposure than is observed for high temperature growth (Fig. 3.8C), suggesting that differences in phosphorylation state for native Mpk1p in these panels might be below the detection threshold. In other assay conditions, we observe clear *MID2*-dependence for native Mpk1p tyrosine phosphorylation in response to calcofluor white and mating factor (not shown). Deletion of *MPK1* results in calcofluor white hypersensitivity, likely due to gross disturbances in cell wall construction. Saliently, overexpression of *MPK1*, like overexpression of *MID2*, also results in a *CHS3*-dependent hypersensitivity to calcofluor white (not shown). This effect is *MID2*-dependent since overexpression of *MPK1* does not bypass the resistance to calcofluor white displayed by *mid2Δ* cells (not shown).

Figure 3.8 Immunoblot analysis of Mpk1p-HA tyrosine phosphorylation. Lanes are loaded with equal amounts of extracts from the strains **a)** TK96 (wild type+pFL44) **b)** TK97 (wild type+pFL44-*MPK1-HA*) **c)** TK93 (*mid2Δ*+pFL44) **d)** TK94 (*mid2Δ*+pFL44-*MPK1-HA*). Cultures exposed to (**A**) α factor (**B**) calcofluor white or (**C**) high temperature growth were harvested at the indicated times and total cell proteins were subject to SDS-PAGE and Western blotting. In the top panel of each pair tyrosine phosphorylation of Mpk1p-HA is detected by anti-phosphotyrosine antibody 4G10. In the second panel of each pair, equal loading of Mpk1p-HA is demonstrated by anti-HA antibody 12CA5.

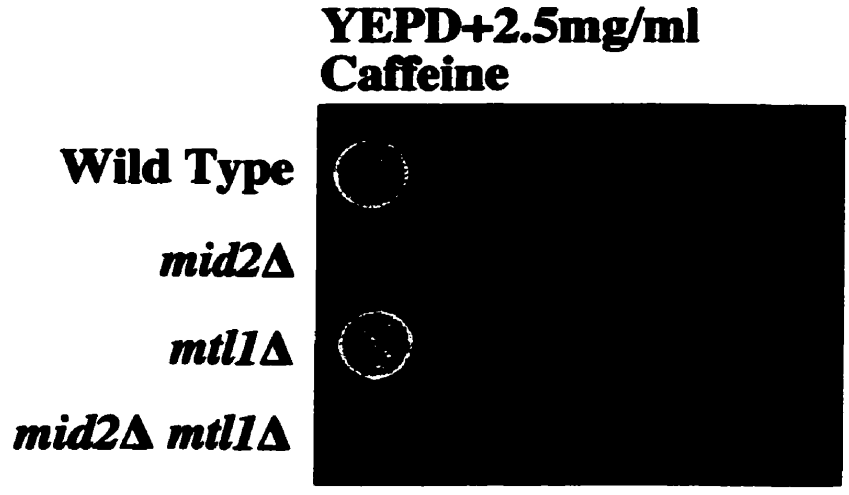

30° Control
Stimulus
A
a b c d a b c d
Mpk1p-HA
Mpk1p
4G10
α factor 3hrs.
Mpk1p-HA
HA11
B
Mpk1p-HA
Mpk1p
4G10
calcofluor white 30 Min.
Mpk1p-HA
HA11
C
Mpk1p-HA
Mpk1p
4G10
37° 3hrs.
Mpk1p-HA
HA11

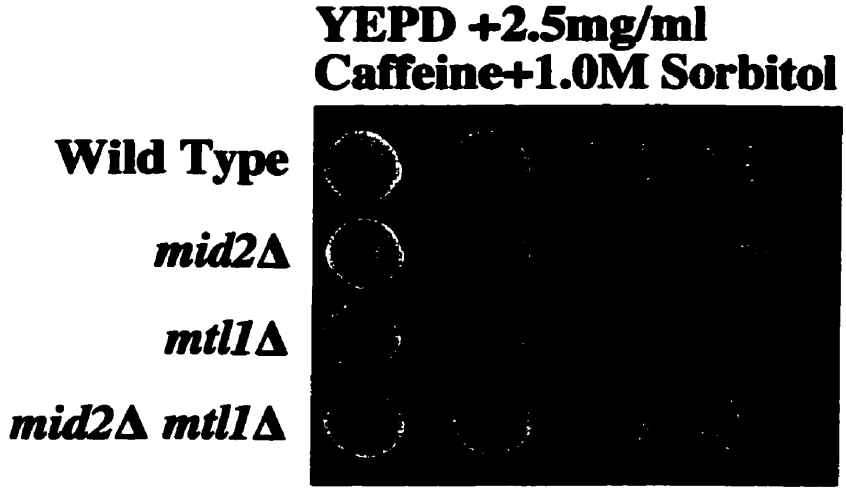
Finally, we examined whether induction of tyrosine phosphorylation of Mpk1p-HA during periods of high temperature stress requires *MID2*. Although *mid2Δ* cells do not have a growth defect at 37°C, induction of tyrosine phosphorylation of Mpk1p-HA was significantly impaired in *mid2Δ* mutants compared to wild type cells (Fig. 3.8C). Together, these observations suggest a role for Mid2p in activation of the Mpk1p MAP kinase cascade under a variety of stress conditions.

Identification of a Mid2p Functional Homolog

The *S. cerevisiae* genome contains a gene, *YGR023W*, which encodes a 551 amino acid residue protein with both structural and amino acid sequence similarity to Mid2p (W.U. Blast V2.0 P(N) value of $1.2e^{-27}$). We will refer to *YGR023W* as *MTL1* (Mid Two Like 1). To initiate the characterization of *MTL1*, we have disrupted its entire open reading frame. Unlike *mid2Δ* cells, *mtl1Δ* mutants in the SEY6210 strain background have no distinguishable phenotype when challenged with temperature extremes, oxidative and osmotic stresses, α factor, calcofluor white or mutation of the *KRE6* or *FKS1* cell wall synthesis genes (not shown). While *mtl1Δ* single mutants are not hypersensitive to caffeine, *mid2Δ* cells are mildly more susceptible than wild type cells, and *mid2Δ mtl1Δ* double mutants show strong sensitivity to this drug. This phenotype appears to be the result of cell lysis since it is suppressible by the inclusion of 1M sorbitol in the growth medium (Fig. 3.9). Caffeine sensitivity of the *mid2Δ mtl1Δ* mutant is also suppressible by overexpression of *WSC2*, however, multicopy *WSC1*, *RHO1*, *PKC1*, *BCK1*, or *MPK1* do not bypass this phenotype. Finally, although *mid2Δ mtl1Δ* double mutants are no more sensitive to α factor than *mid2Δ* cells (not shown), high copy expression of *MTL1* from either its own promoter or the *ADH1* promoter is able to suppress the caffeine (not shown) and α factor sensitivity of *mid2Δ* cells (Fig. 3.7), suggesting that *MTL1* is a functional gene, and that Mtl1p might have related or overlapping activity with Mid2p.

Figure 3.9 Caffeine sensitivity of mutants lacking *MID2* and/or *MTL1*. 10-fold serial dilutions of liquid culture medium containing wild type, *mid2Δ*, *mtl1Δ* and *mid2Δ mtl1Δ* strains were spotted onto plates containing either 2.5mg/ml caffeine or 2.5mg/ml caffeine+1M sorbitol and incubated for 72 hours at 30°C then photographed.


YEPD+2.5mg/ml
Caffeine
Wild Type
mid2Δ
mtl1Δ
mid2Δ mtl1Δ


YEPD +2.5mg/ml
Caffeine+1.0M Sorbitol
Wild Type
mid2Δ
mtl1Δ
mid2Δ mtl1Δ

3.5 Discussion

Mid2p is an O-Mannosylated, Plasma Membrane Protein

In this work, we offer evidence suggesting that Mid2p could potentially sense cell wall state, and act to initiate a cellular response involving both chitin synthesis and the *PKC1-MPK1* cell integrity pathway. Mid2p is a Type I integral membrane protein which localizes to the plasma membrane and contains a large, extensively O-mannosylated extracellular serine/threonine-rich region. Removal of the serine/threonine-rich region does not affect the targeting of Mid2p, however ΔS/T-Mid2p is unable to complement *mid2Δ* cells, suggesting that this domain is required for Mid2p activity. This observation contrasts with the findings for Gas1p and Kre1p, two GPI-anchored proteins involved in cell wall synthesis, where deletion of the serine/threonine-rich sequences does not greatly affect function of these proteins (Boone *et al.* 1991, Gatti *et al.* 1994). O-linked mannosylation could cause the extracellular region to adopt a stiff and extended conformation (Jentoft 1990) that reaches from the plasma membrane towards the cell wall, perhaps interacting with it. In Mid2p, this region is unlikely to play a direct enzymatic role since it is largely composed of repetitive non-complex amino acid sequence, and it lacks any similarity to known enzymatic motifs. An intriguing possibility is that the extracellular domain could act as a sensor of cell wall state.

MID2 Interacts with Genes Required for Cell Wall Construction and Cell Wall Integrity Signaling

We isolated *MID2* as an activator of the Skn7p transcription factor. Skn7p appears to affect a number of cellular processes, including cell wall biosynthesis. *SKN7* was identified by Brown *et al.* (1993) as a high copy suppressor of the *kre9Δ* mutant and it was

later demonstrated that Skn7p might function in parallel with Pkc1p since overexpression of *SKN7* can suppress the lysis defect of *pkc1Δ* mutants, and *skn7Δ pkc1Δ* cells are inviable, even on medium containing osmotic support (Brown *et al.* 1994). Recently, Alberts *et al.* (1998) have shown that Rho1p and Skn7p physically interact, and that the domain in Skn7p that mediates this interaction is important for activity. It is not clear how Mid2p might stimulate Skn7p transcriptional activity. Since *RHO1* appears to have interactions with both *SKN7* and *MID2*, one avenue of future research will be to examine whether Rho1p might mediate a Mid2p-Skn7 interaction.

Deletion of *MID2* causes significant changes in growth rate or viability for a variety of cell wall synthesis mutants. It is curious that for the β-1,6-glucan mutants examined, loss of *MID2* increases growth rate, while for the β-1,3-glucan mutant *fks1Δ*, deletion of *MID2* causes inviability. One possibility is that reduction of supplementary chitin levels caused by absence of Mid2p might be a contributing factor. *fks1Δ* mutants seem to depend heavily on enhanced chitin synthesis to maintain viability since they are supersensitive to Nikkomycin Z, a chitin synthase inhibitor (el-Sherbeini and Clemas 1995). Conversely, there is some evidence that attenuation of the chitin synthesis stress response might actually be beneficial in cells lacking proper β-1,6-glucan synthesis, specifically in *kre9Δ* mutants (Nagahashi *et al.* 1998). Consistent with this hypothesis, overexpression of *MID2*, which causes hyperaccumulation of chitin, is deleterious to β-1,6-glucan mutants, but has little effect on the β-1,3-glucan-deficient mutant *fks1Δ*. A second possibility is that Mid2p might influence the activity of a distal signaling element in the cell integrity pathway such as Rho1p, which in turn may directly affect cell wall synthesis. Rho1p activates the Fks1p glucan synthase, and presumably is also required for the activation of Fks2p. In the absence of *FKS1*, β-1,3-glucan is severely disrupted, although cells can survive due to residual β-1,3-glucan synthase activity provided by Fks2p. If activation of Rho1p is diminished, perhaps by the loss of *MID2*, it is conceivable that β-1,3-glucan synthesis in *fks1Δ* mutants could be disrupted to a point that results in cell death.

Screens to identify genes which interact with *MID2* uncovered a relationship between *MID2* and several components of a pathway known to promote cell integrity and polarized growth. Caffeine sensitivity of the *mid2Δ* and *mid2Δ mtl1Δ* mutants is suppressed by overexpression of *WSC2*, high copy *WSC1* or *PKC1* suppresses *mid2Δ fks1Δ* synthetic lethality, and multicopy *PKC1*, *RHO1*, *WSC1*, and *WSC2* suppresses lethality in *mid2Δ* MAT **a** cells exposed to α factor. Additionally, *mid2Δ wsc1Δ* mutants are inviable without osmotic support, and exhibit defects similar to those observed in *pkc1Δ* mutants. Finally, we find that during shmoo formation, high temperature growth, or exposure to calcofluor white, tyrosine phosphorylation of Mpk1p, a downstream target of Pkc1p, is reduced in *mid2Δ* cells. Interestingly, although *mid2Δ* mutants have reduced tyrosine phosphorylation of Mpk1p in response to high temperature growth, these cells are not deficient for growth at high temperature. It is possible that there is sufficient remaining Mpk1p activity to allow cells to survive high temperature stress or that some other mechanism is able to compensate for under activation of this pathway. Strain differences may also play some role here since we do not observe high temperature sensitivity in *wsc1Δ* mutants as reported by Verna *et al.* (1997).

Although *mid2Δ* cells have no apparent vegetative growth defects, shmoo formation, mutation of cell wall synthesis genes, and exposure to calcofluor white cause *mid2Δ* mutants to manifest phenotypes. One possibility is that Mid2p, and perhaps members of the *WSC* family act indirectly, through effects on cell wall structure, to activate the *PKC1-MPK1* pathway. In an alternative model, Mid2p might sense cell wall stress, and directly act to increase activity in the cell integrity pathway to counteract damage. Under non-stressed conditions, Mid2p activity might be low, or not be required, and Wsc1p and its homologs may be largely responsible for Pkc1p activation. However, under circumstances of cell wall stress, in the absence of Mid2p, the cell integrity machinery would be unresponsive, and would continue to function at a level more appropriate for low or non-stress situations (Fig. 3.10).

This model explains why some of the phenotypes observed in *mid2Δ* mutants contrast with those displayed by other mutants in the cell integrity pathway. For example, mutants such as *wsc1/2/3Δ*, *pkc1Δ*, *bck1Δ*, or *mpk1Δ* are prone to cell lysis in the absence of osmotic support. These genes are required for normal function of the cell integrity pathway under all growth conditions. In their absence, construction and maintenance of the cell wall is defective, making the wall highly susceptible to damage. *mid2Δ* mutants have apparently normal cell walls when grown in ordinary conditions. However, in stress situations, such as exposure to calcofluor white or α factor, *mid2Δ* mutants have an attenuated response compared to wild type cells as indicated by the reduced amount of new chitin synthesized, and by the reduced extent of tyrosine phosphorylation on Mpk1p.

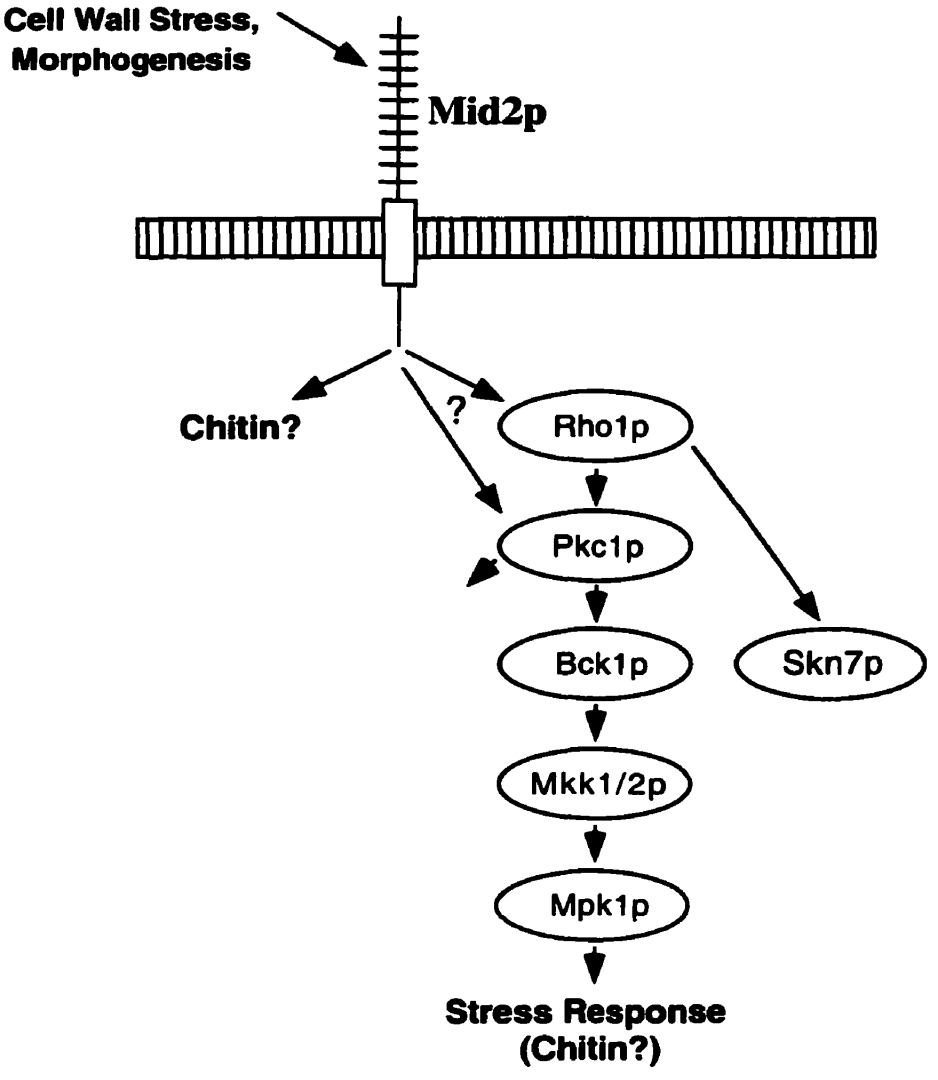
Preliminary investigation of *MTL1*, the only *S. cerevisiae* gene encoding a protein with significant sequence similarity to Mid2p, revealed that it may share a common function with *MID2*. Although *mtl1Δ* mutants do not display many of the phenotypes that *mid2Δ* cells do, sensitivity to caffeine is much greater in *mid2Δ mtl1Δ* double mutants than either single mutant. Additionally, multicopy *MTL1* can suppress α factor sensitivity of *mid2Δ* cells. Further research may reveal whether Mtl1p is required for responding to different stresses than Mid2p, signals to a different pathway than Mid2p, or is important under different physiological conditions than were used in this study.

The effect of Mid2p on chitin synthesis depends ultimately on Chs3p, since overexpression of *MID2* in *chs3Δ* mutants cannot confer hypersensitivity to calcofluor white. We suggest two ways in which Mid2p might affect chitin synthesis. Mid2p might directly interact with the chitin synthase complex, increasing its activity during stress periods. Alternatively, the activity of Chs3p might be regulated by a downstream target of Mid2p, such as Mpk1p.

Cells face a demanding variety of stresses in their natural environments and must respond accordingly. Our results provide new insights into the processes by which yeast cells sense and respond to threats against their physical integrity. Mid2p has been

identified as a putative sensor of cell wall state that is required for stimulation of activity in the *PKC1-MPK1* MAP kinase pathway under conditions of cell wall stress. Currently, the physiological roles of most serine/threonine-rich membrane proteins are poorly defined. An interesting possibility is that, as a class, these proteins act as sensors for a range of environmental stresses and mediate a variety of cellular processes.

Figure 3.10 Model of Mid2p activity. Mid2p responds to cell wall stress and morphogenesis by activating the cell integrity pathway and increasing chitin synthesis


Cell Wall Stress,
Morphogenesis
Mid2p
Chitin?
?
Rho1p
Pkc1p
Bck1p
Skn7p
Mkk1/2p
Mpk1p
Stress Response
(Chitin?)

CHAPTER 4

Yeast Mid2p is a Positive Regulator of the Rho1p G-Protein

4.1 Abstract

The *Saccharomyces cerevisiae* gene *MID2* encodes a putative cell surface sensor protein that modulates activity in the PKC1-MPK1 cell integrity pathway. Overexpression of *MID2* is able to bypass a *tor2* mutant that is deficent in activation of the *RHO1* GTPase. Loss of *MID2* is capable of suppressing a cold sensitive growth defect in cells lacking *SAC7*, the gene encoding a Rho1p GTPase-activating protein. Conversely, overexpression of *MID2* in the *sac7Δ* mutant causes a severe growth defect at all temperatures. Absence of *MID2* is lethal in a strain lacking Rom2p, the major GDP-GTP exchange factor for Rho1p, while overexpression of *MID2* in the same strain is partially able to bypass growth defects. Elevated expression of *MID2* stimulates GDP-GTP exchange activity towards Rho1p. We propose that Rho1p GDP-GTP exchange activity is promoted by Mid2p.

4.2 Introduction

Mid2p has characteristics of a cell wall sensor protein, and modulates activity in the *PKC1-MPK1* cell integrity pathway (Ketela *et al.* 1999, Rajavel *et al.* 1999). Genetic interactions between *MID2* and a number of genes in the cell integrity pathway suggest that Mid2p's influence on this pathway may occur at a very early step in pathway signaling. Loss of *MID2* is lethal in strains lacking *WSC1*, a gene that encodes another protein proposed to function upstream of the cell integrity pathway (Gray *et al.* 1997, Jacoby *et al.* 1998, Verna *et al.* 1997), while *mid2Δ pkc1Δ* mutants are no more severe than *pkc1Δ* cells. Also, overexpression of various genes whose products function within the cell integrity pathway are able to suppress pheromone induced death in *mid2Δ* cells. Finally, Mid2p is required for the stress-induced activation of the Mpk1 MAP kinase (Ketela *et al.* 1999, Rajavel *et al.* 1999).

How, and at what step Mid2p influences signaling in the PKC1-MPK1 pathway is not yet clear. However, a number of observations suggest that Mid2p might be a regulator of Rho1p activity. Firstly, overexpression of *RHO1* is able to suppress some *mid2Δ* phenotypes, while overexpression of *MID2* is not able to compensate for loss of *RHO1*. Secondly, overexpression of *RHO1* is significantly better at suppressing the pheromone-induced death of *mid2Δ* mutants than even a hyperactive allele of *PKC1*. Finally, a similarity exists between cells lacking Mid2p and cells depleted for Rho1p activity in that pheromone-induced death in *mid2Δ* mutants, apparently a lysis defect, is not suppressible by the addition of sorbitol, an osmotic stabilizer to growth medium. *rho1Δ* mutants also display a cell lysis phenotype that is not suppressible by addition of osmotic stabilzer (Nonaka *et al.* 1995). Presumably, this is due to Rho1p performing several functions in addition to activating Pkc1p, such as stimulating ß-1,3-glucan synthesis, and coordinating actin cytoskeleton assembly (Qadota *et al.* 1996, Drgnova *et al.* 1996, Imamura *et al.* 1997, Helliwell *et al.* 1998). Cells lacking *PKC1* or any of the members of the *MPK1* MAP

kinase cascade are also prone to cell lysis, however, the phenotype is largely reversible by addition of sorbitol.

To test if Mid2p is an upstream regulator of Rho1p, we looked for genetic interactions between *MID2* and genes known to regulate the activity state of Rho1p. In addition, we measured GEF activity towards Rho1p in extracts prepared from cells that were either lacking Mid2p or contained increased levels of Mid2p.

4.3 Materials and Methods

Construction of Strains

The *SAC7* open reading frame was deleted by PCR-based gene disruption. The *KANMX2* marker cassette (Wach *et al.* 1994) flanked by 45bp of *SAC7* sequence on each end was amplified using the oligonucleotides *sac7Δup* (ATGCCAAATAATACTCTTAAACAAGGCTCCAAAATTGAAAATGTTGATATCAAG CTTGCCTCG) and *sac7Δdown* (TCAAGCCCTGTTAGCAGAGCCTGAACGGCTTGTTAGTCTTTGAAAGTCGACAC TGGATGGCGG). The resulting PCR product was transformed directly into strain SEY6210 using a high efficiency, lithium acetate-based method (Gietz and Schiestl 1991). Verification of *KANMX2* integration into the *SAC7* locus was obtained by PCR analysis of Geneticin-resistant yeast colonies using the oligonucleotides *sac7Δtest* (CGAGCTTCCAATTTGATTCC) and *KANMX2internalreverse* (CAACAGGCCAGCCATTAC). The *ROM2* open reading frame was deleted by PCR-based gene disruption using the *GFP-HIS3* marker cassette (Niedenthal *et al.* 1996) with the oligonucleotides *rom2Δup* (ATGAGCGAAACCAACGTTGACAGCTTAGGGGACAGAAATGACATAATGAGTA AAGGAGAAGAAC) and *rom2Δdown*

(TTAACCCCAGAAATCTAACGACGCAACTGTATCATACCCGCGATAGCGCGCCT CGTTCAGAATG) essentially as described deletion of *SAC7*. Correct integration of the *rom2Δ::GFP-HIS3* cassette in candidate histdine prototrophic colonies was verified by PCR using the oligonucleotides *rom2Δtest* (GTGTTAGGATCTACAGGG) and *GFP-HIS3internal reverse* (GTATAGTTCATCCATGCC) to amplify a specific fragment. Deletion of *ROM1* with the *KANMX2* cassette was accomlished using the oligonucleotides *rom1Δup* (ATGAATAGTAATGAACTGGATCTAAGAAATAAATATTTTTATGAGGATATCAAG CTTGCCTCGTC) and *rom1Δ down* (TCAAAAATTTAACAGTTCGATAATTTCAAATCCTTGAGGATCCTCGTCGACACT GGATGGCGGCG). Verification of putative *rom1Δ::KANMX2* strains was accomplished by PCR of genomic DNA using the oligonucleotides *rom1Δ test* (GCGCTCCAGTACTCCAAT) and *KANMX2internalreverse* to yield a specific fragment. Deletion of *MID2* was described in Ketela *et al.* (1999), and the *tor2*^{ts} strain, and HA-tagged *RHO1* was obtained from M. Hall.

Measurement of GEF Activity

Measurement of GEF activity towards Rho1p was performed essentially as described by Bickle *et al.* (1998) with modifications. All procedures were performed at 4°C unless otherwise noted. Cell cultures (50mL) grown to mid log phase (OD_{600}=0.5-0.8) were harvested, washed once in GEF EXTRACTION BUFFER (20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 2.5mM EDTA, 1mM DTT, 1mM PMSF) and then resuspended in 500µl GEF EXTRACTION BUFFER. Cell lysates were prepared by adding 200 µl glass beads and alternately vortexing and resting on ice for 30 second periods for a total of 8 min. Cell walls and debris were removed by centrifuging at 500xg for 8 min. Total protein concentration was determined using the Biorad™ microassay procedure. Cell extracts from

cultures containing HA-Rho1p were prepared by the same method, using GTPase EXTRACTION BUFFER (50mM Tris-HCl, 100mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM PMSF). For each GEF assay to be performed, 200µg of cell extract was precleared with 25µl of GammaBind protein G Sepharose (Amersham-Pharmacia). 2µl of HA11 antibody (Babco) was added to precleared lysates and incubated for 1 hour with rotation. 50µl of GammaBind G protein G Sepharose were then added, and tubes were incubated for a further 2 hours with rotation. Immune complexes were washed 5 times with IP WASH BUFFER (50mM Tris-HCl pH 7.5, 500mM NaCl, 1mM EDTA, 0.5% NP-40, 0.5% Triton X-100, 1mM PMSF), once with GEF EXTRACTION BUFFER, and then resuspended in 50µl of GEF ASSAY BUFFER (GEF EXTRACTION BUFFER +1mg/ml di-myristoylphosphotidyl choline). The reaction was initiated by the addition of 150µg of total protein (contained in a volume of 40µl) of extracts to assayed for GEF activity, and 10µl of 10µM GTP S^{35} γS (Amersham). Reactions were incubated at 25°C for 10 min. with occasional mixing, and then stopped by the addition of 1ml of ice cold STOP BUFFER (20mM Tris-HCl pH 7.5, 25mM $MgCl_2$, 100mM NaCl). Beads were washed once with 1ml of STOP BUFFER then incubated in 500µL of 0.5M acetic acid pH 3.0 for 5 min to release proteins from the sepharose. 250µl of the supernatant was transfered to a scintillation vial containing 5ml of Ecolite™ (ICN), and then counted. Rho1p-specific GEF activity was calculated by subtracting the GEF activity measured in reactions performed without Rho1p from the activity measured in reactions performed with Rho1p.

4.4 Results and Discussion

MID2 Counteracts SAC7 Activity

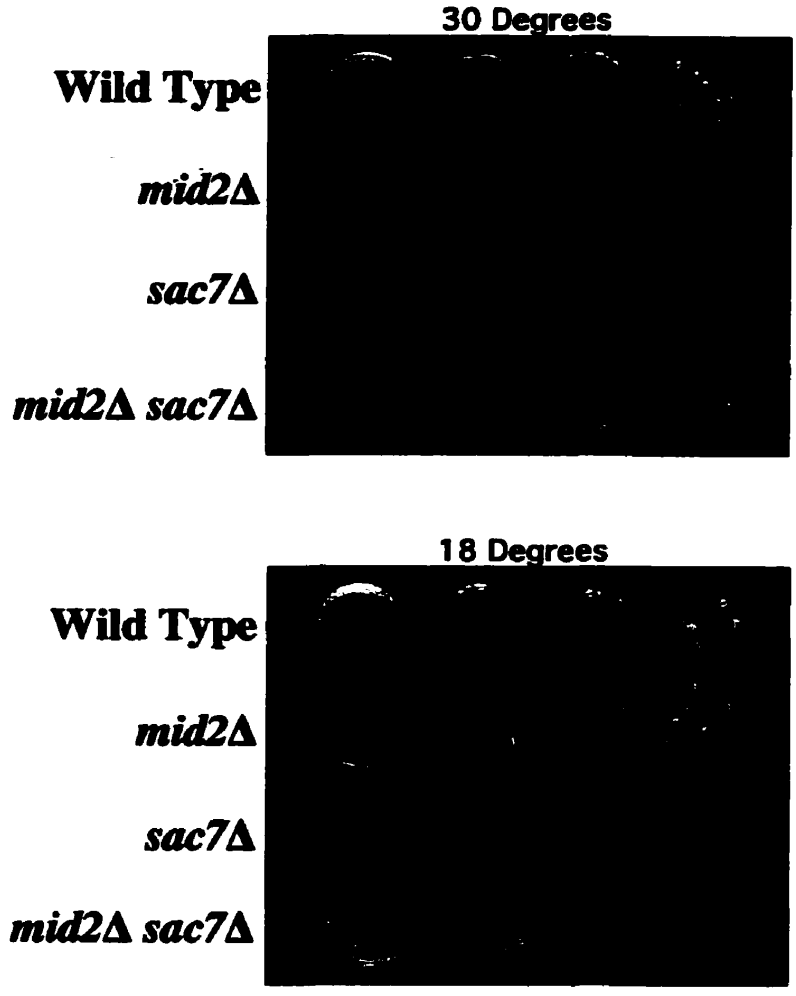
SAC7 (Suppressor of Actin) was first identified as an extragenic suppressor of growth and actin cytoskeleton defects in the actin mutant *act1-4* (Dunn and Shortle, 1990).

Subsequently, Schmidt *et al.* (1997) demonstrated that Sac7p is a GAP for Rho1p. Strains lacking *SAC7* grow well at 30°C, but become inviable, and display abnormal actin cytoskeleton assembly at 18°C.

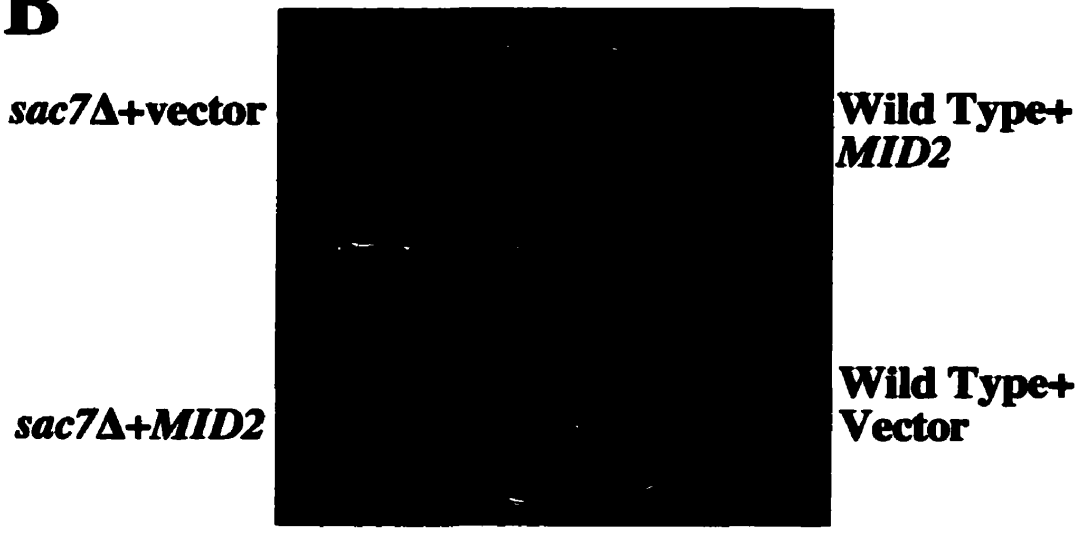
The possibility that Mid2p might affect Rho1p activity led us to examine the effect of altered dosage of *MID2* in strains lacking *SAC7*. A *sac7Δ/mid2Δ* heterozygous diploid strain was constructed, sporulated, and the resulting strains were phenotypically analyzed (Fig. 4.1A). At 30°C and 37°C (not shown), wild type, *mid2Δ*, *sac7Δ* and *mid2Δ sac7Δ* strains grow equally well. At reduced temperature (18°C), while wild type and *mid2Δ* cells grow robustly, *sac7Δ* cells are significantly impaired in growth. Notably, the *sac7Δ mid2Δ* double mutant grows substantially better at 18°C than the *sac7Δ* single mutant. Conversely, overexpression of *MID2* in *sac7Δ* mutants is highly deleterious to cell growth at 30°C (Fig. 4.1B) and 37°C (data not shown). Restoration of low temperature growth in *sac7Δ* mutants can also be achieved by deletion of *ROM2* in *sac7Δ* cells (Schmidt *et al.* 1997), presumably because Rom2p is a GEF for Rho1p, and its activity antagonizes the GAP activity of Sac7p. Together, these observations suggest that Mid2p activity may oppose that of Sac7p in vivo.

Figure 4.1 Genetic interactions between *MID2* and *SAC7*. **A)** Loss of *MID2* is able to bypass the low temperature slow growth defect of *sac7Δ* mutants. **B)** Overexpression of *MID2* is toxic to cells lacking *SAC7*.

A


30 Degrees
Wild Type
mid2Δ
sac7Δ
mid2Δ sac7Δ
18 Degrees
Wild Type
mid2Δ
sac7Δ
mid2Δ sac7Δ

B


sac7Δ+vector
sac7Δ+MID2
Wild Type+ MID2
Wild Type+ Vector

High Copy *MID2* Suppresses A *tor2*^{ts} Mutant

The putative phosphotidylinositol (PI) kinase Tor2p has two indispensable functions (Hall *et al.* 1996, Helliwell *et al.* 1994, Kunz *et al.* 1993, Zheng *et al.* 1995). One activity of Tor2p is redundant with Tor1p, and is required for early G1 progression in response to nutrient availability. The second function of Tor2p is to activate Rom2p, and thereby stimulate Rho1p activity. A mutant allele of Tor2p, *tor2*^{ts}, causes disruption in actin cytoskeleton assembly and diminished GEF activity towards Rho1p (Schmidt *et al.* 1996). At nonpermissive temperature (37°C), *tor2*^{ts} mutants are inviable, presumably due to insufficient activation of Rho1p. Since Mid2p counteracts Sac7p, and possibly thereby is a positive regulator of the switch apparatus regulating Rho1p, we examined if high dosage of Mid2p could bypass the *tor2*^{ts} phenotype. At the nonpermissive temperature, *tor2*^{ts} cells are inviable, while *tor2*^{ts} mutants carrying multicopy *MID2* are viable (Fig. 4.2). Survival of the *tor2*^{ts} mutant is further increased by driving *MID2* expression with the strong *ADH1* promoter. This finding suggests that Mid2p may act in parallel with Tor2p to activate Rho1p.

High Copy *MID2* Increases Rho1p GEF Activity

Since Rho1p is functional when it is in its GTP-bound state, the level of Rho1p activity can be inferred from the amount of GEF activity towards it that is present in cell extracts (Ozaki *et al.* 1996, Schmidt *et al.* 1997). To test if Mid2p can up-regulate Rho1p activity, we measured the GDP-GTP exchange activity towards Rho1p in extracts prepared from strains containing different gene dosages of *MID2* (Fig 4.3). We found that although loss of *MID2* does not affect Rho1p GEF activity, high expression of *MID2* increases GEF

activity to ~135% of the level seen in wild type cells. It is not suprising that *mid2Δ* mutants cultured in medium under ideal growth conditions are not reduced for Rho1p GEF activity since *mid2Δ* mutants do not display any phenotype in these conditions. However, the finding that Rho1p GEF activity is increased in cells overexpressing *MID2* corresponds well with the observation that high copy *MID2* causes a mild retardation of growth in wild type cells (Ketela *et al.* 1999), and results in substantial thickening of the cell wall (Marcoux *et al.* 1998, D. Pallota, personal communication), probably as a result of increased synthesis, or altered levels of chitin cross-linking of cell wall components (Ketela *et al.* 1999).

MID2 Genetically Interacts with *RHO1* GEFs

Since positive regulation of Rho1p activity is provided by GEFs, we sought to uncover interactions between *MID2* and the genes encoding GEFs for Rho1p; *ROM1* and *ROM2*. Cells lacking *ROM1* do not display obvious phenotypes, while *rom2Δ* mutants are slow growing, prone to lysis at high temperature (Ozaki *et al.* 1996), and display abnormal mating projection morphology (Manning *et al.* 1997), suggesting that *ROM2* encodes the major *RHO1* GEF activity. Cells lacking both *ROM1* and *ROM2* are inviable, and have the same, small-budded terminal phenotype as cells lacking Rho1p, indicating that Rom1p and Rom2p have overlapping activity.

mid2Δ/rom1Δ and *mid2Δ/rom2Δ* heterozygous diploids were constructed, sporulated, and the resulting meiotic products were subjected to tetrad dissection and phenotypic analysis. We analyzed 27 tetrads, and found that in all tetra type and non parental ditype tetrads, colonies predicted to consist of *mid2Δ rom2Δ* mutants failed to form, suggesting that *mid2Δ rom2Δ* mutants are inviable (Fig. 4.4A). Microscopic examination revealed that cells derived from *mid2Δ rom2Δ* spores divide one to three times before ceasing growth. This phenotype is not changed by addition of sorbitol, or altered incubation temperature (18°C and 37°C) (data not shown).

The inability to recover *mid2Δ rom2Δ* colonies from tetrad dissection is not the result of a germination defect since *mid2Δ rom2Δ* mutants carrying *URA3* centromeric plasmids bearing either *MID2* or *ROM2* become inviable when incubated on medium containing 5-FOA, which forces loss of plasmids bearing the *URA3* marker (Fig. 4.4B). The possibility that inviability of *mid2Δ rom2Δ* mutants is due to insufficient Rho1p activity is supported by the observation that high copy expression of RHO1 is capable, albeit weakly, of restoring viability to these mutants (Fig. 4.4B). The reported high temperature sensitivity of *rom2Δ* mutants (Ozaki *et al.* 1996) is not pronounced in the SEY6210 strain, so we were unable to test if overexpression of *MID2* is able to bypass this phenotype.

Rho1p GEF activity can be stimulated by cell wall defects, in a process that requires *ROM2*, but is independent of *TOR2*. Since we have previously postulated that Mid2p is a sensor of cell wall state, and have demonstrated here that Mid2p is an activator of Rho1p, and can bypass a *tor2*^{ts} mutant, it might be expected that Rom2p mediates the activation of Rho1p by Mid2p. However, if this were true, the growth defect of *mid2Δ rom2Δ* mutants should be no worse than that displayed by *rom2Δ* mutants. The inability of *mid2Δ* cells to survive incubation in the presence of exogenously added mating pheromone appears to be the result of insufficient Rho1p activity since high copy expression of *RHO1* largely suppresses this phenotype (Ketela *et al.* 1999). If Mid2p activation of Rho1p required Rom2p, we would expect that *rom2Δ* mutants to be mating pheromone hypersensitive. However, although *rom2Δ* mutants have morphological defects in mating projection formation (Manning *et al.* 1997), they do not become inviable in the presence of pheromone like *mid2Δ* mutants do. Also, we found that overexpression of *ROM2* is not able to suppress pheromone-induced death in *mid2Δ* mutants. Finally, high copy expression of *MID2* is partially able to suppress a *tor2*^{ts} mutant that is deleted for *ROM2* (P. Delley, personal communication, data not shown). Togther, these observations suggest that Rom2p does not mediate interaction between Mid2p and Rho1p.

mid2Δ rom1Δ cells do not have a growth defect on YEPD medium. Because the double mutant is no more severe than either single mutant, it might be possible the Rom1p functions downstream of Mid2p. If this scenario were true, we might expect *mid2Δ* and *rom1Δ* cells to have similar phenotypes. However, we found that unlike *mid2Δ* cells, *rom1Δ* cells are not hypersensitive to mating pheromone and are hypersensitive, not resistant to calcofluor white. In addition, although *mid2Δ* mutants are sensitive to caffeine, *rom1Δ* cells are not sensitive to this drug, and *mid2Δ rom1Δ* mutants are not more sensitive than *mid2Δ* cells.

Evidence presented here suggests that Mid2p is a regulator of Rho1p GEF activity, and acts in parallel to Tor2p. However, Mid2p influence on Rho1p appears to occur independently of the known Rho1p GEFs Rom1p and Rom2p. It is not clear then how Mid2p affects Rho1p. One possibility is that Mid2p directly acts as a GEF for Rho1p. However, this scenario seems unlikely for two reasons. Firstly, the cytoplasmic tail of Mid2p contains no region of homology to a DH domain. The DH domain of Rom2p is necessary and sufficient to stimulate GDP dissociation from Rho1 (Ozaki *et al.* 1996). Secondly, attempts to detect physical interaction between Mid2p and Rho1p using the two-hybrid method have been unsuccessful (R. Green, personal communication). A second possibility is that one or more additional proteins mediate interaction between Mid2p and Rho1p. An intermediary protein need not physically link Rho1p and Mid2p, but could instead perform a different function such as synthesize a cofactor required for Rho1p stimulation.

Figure 4.2 Overexpression of *MID2* bypasses mutants lacking Tor2p activity. Wild type and *tor2*^{ts} strains containing either a control vector, or high copy *MID2* were spotted in 10-fold serial dilutions on selective medium and incubated at 30°C and 37°C.

30 Degrees

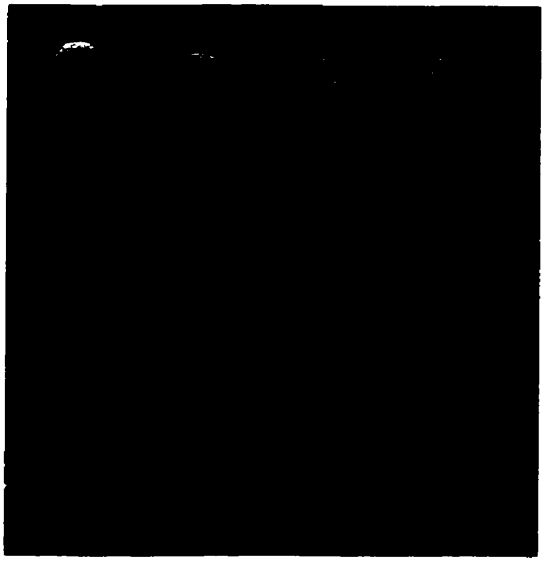
Wild Type +vec

Wild Type +*MID2*

tor2^{ts}+vec

tor2^{ts}+426-*MID2*

tor2^{ts}+pVT-*MID2*



37 Degrees

Wild Type +vec

Wild Type +*MID2*

tor2^{ts}+vec

tor2^{ts}+426-*MID2*

tor2^{ts}+pVT-*MID2*

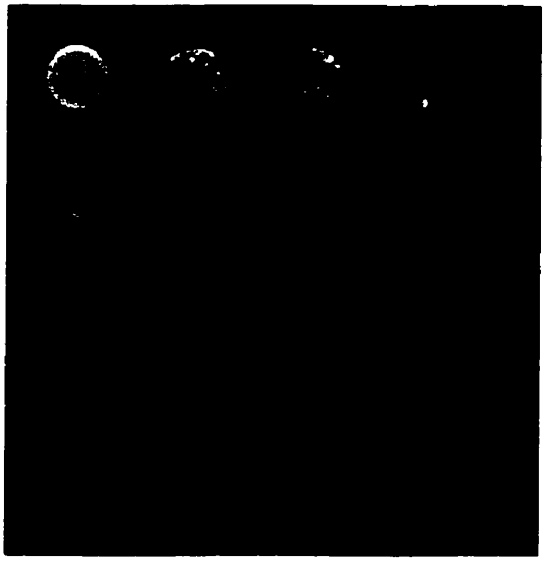


Figure 4.3 Mid2p stimulates Rho1p GEF activity. Immuno-purified Rho1p-HA was incubated with GTP γ^{35}S, and cell extracts obtained from strains either lacking *MID2* or containing high copy *MID2*. Changes in GEF activity are expressed as % change from wild type (100%).

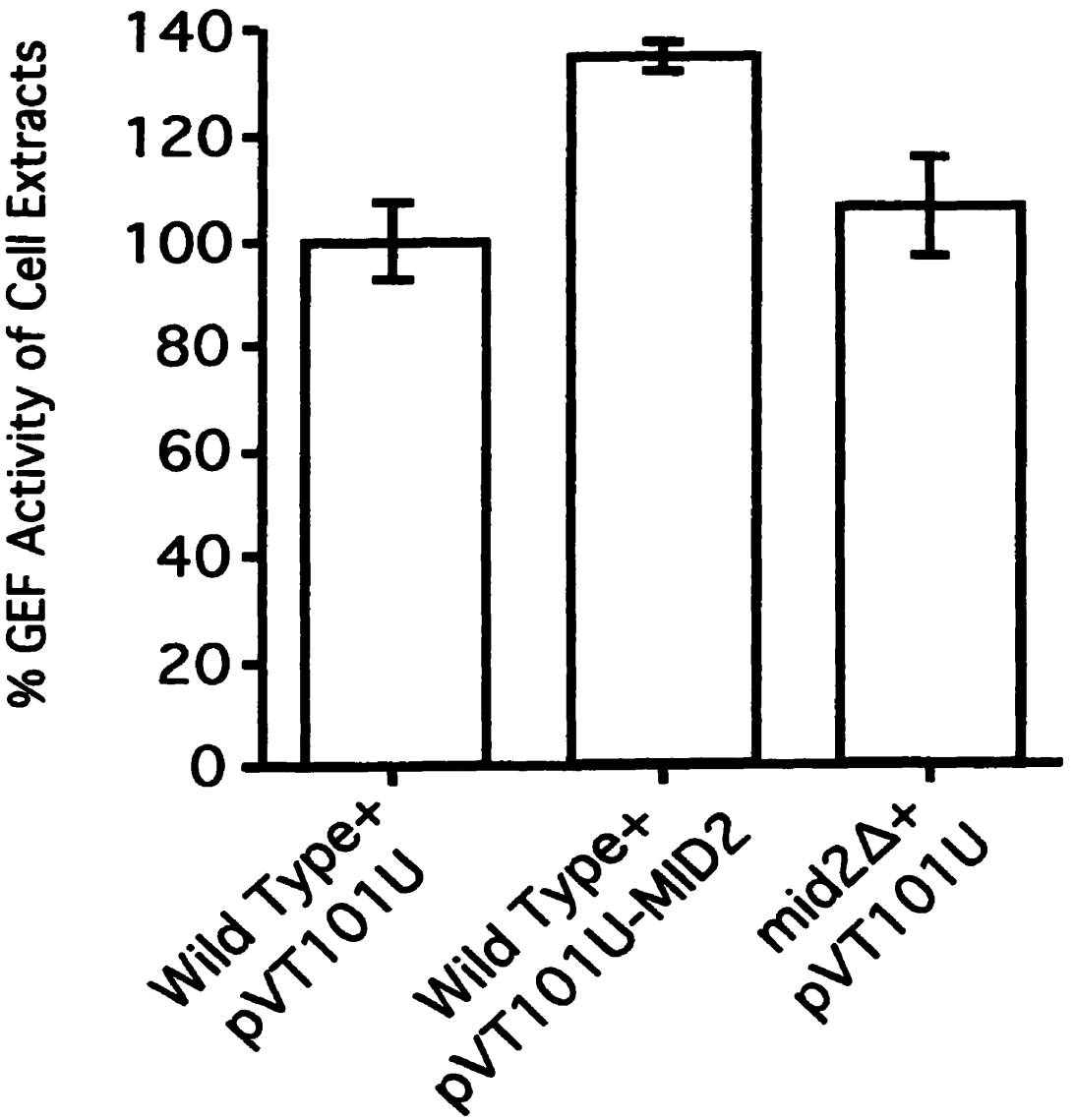
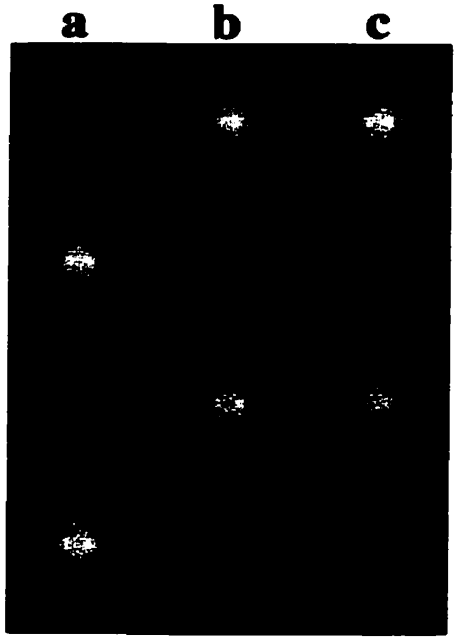
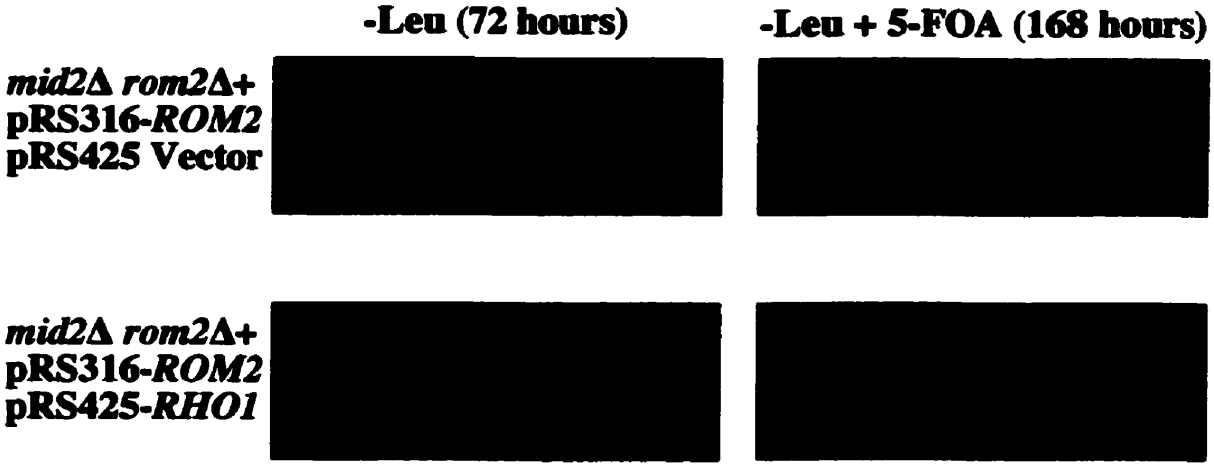

% GEF Activity of Cell Extracts
140
120
100
80
60
40
20
0
Wild Type+ pVT101U
Wild Type+ pVT101U-MID2
mid2Δ+ pVT101U

Figure 4.4 *MID2* genetically interacts with *ROM2*. **A**) Three independent tetra-type tetrads (a-c) derived from *mid2Δ rom2Δ* heterozygous diploids demonstrate that mutants lacking both *MID2* and *ROM2* are inviable. Within each tetrad, the genotypes of the two large colonies are wild type and *mid2Δ*, the genotype of the slow growing colony is *rom2Δ*, and *mid2Δ rom2Δ* mutants stop growing after 1-3 cell divisions, and fail to form colonies. **B**) High copy expression of *RHO1* suppresses the inviability of *mid2Δ rom2Δ* mutants. *mid2Δ rom2Δ* mutants containing pRS316-*ROM2* and either a vector-only control or pRS425-*RHO1* were struck on selective medium in which 5-FOA (to eliminate pRS316-*ROM2*) was either present or absent. Petri plates were incubated at 30°C.

A


a
b
c

B


-Leu (72 hours)
-Leu + 5-FOA (168 hours)
mid2Δ rom2Δ+
pRS316-ROM2
pRS425 Vector
mid2Δ rom2Δ+
pRS316-ROM2
pRS425-RHO1

Chapter 5

Summary and Conclusion: The Roles of Skn7p and Mid2p in Cell Stress Signaling

5.1 Summary and Conclusion

This thesis summarizes the results obtained from studies aimed at determining the upstream regulatory apparatus for Skn7p, and the physiological role for Mid2p. From these studies, new understanding was gained on two different stress response pathways in the model organism *Saccharomyces cerevisiae*.

The *SKN7* gene was initially identified and characterized by Brown *et al.* (1993a, 1994). Evidence from these studies suggested that Skn7p was an orthodox two component response regulator protein, and that it was modulated by histidine kinase activity. The identity of Skn7p's cognate kinase, and the conditions under which the kinase stimulated Skn7p were unknown. A role for Skn7p in the oxidative stress response had been uncovered by Krems *et al.* (1996) and Morgan *et al.* (1997), but Skn7p function in this stress response occurs independently of histidine kinase activity.

Sln1p had been identified as a histidine kinase, and as a regulator of the osmotic stress response (Ota *et al.* 1993, Maeda *et al.* 1994). Until the completion of the yeast genome sequencing project, it was not known if *S. cerevisiae* contained another histidine kinase-encoding gene. Since attempts to identify a cognate histidine kinase for Skn7p via genetic screens had been unsuccessful, I directly tested, via a reporter system, whether the osmosensing circuit consisting of Sln1p, Ypd1p and Ssk1p was involved in Skn7p activation. This experiment demonstrated that Skn7p is a component of the *S. cerevisiae* osmoresponsive phosphorelay, and that Sln1p modulates Skn7p via Ypd1p. This was the first reported instance of a branched two component relay system in a eukaryotic species. These genetic data were later confirmed by biochemical means, that directly demonstrated phosphotransfer from Sln1p to Ypd1p to Skn7p (R. Stewart, personal communication, Li *et al.* 1998).

Additional genetic experiments suggested that Skn7p plays a role in regulating activity in the HOG MAP kinase cascade. Cells lacking either *SKN7* or *PTC1* grow well,

however mutants lacking both genes had severe growth defects, and hyperaccumulation of glycerol. These defects can be bypassed by deleting either *PBS2* or *HOG1*. Since Skn7p is a transcription factor, it is not likely to be a direct regulator of Pbs2p or Hog1p kinase activity, but instead, Skn7p likely regulates the transcription of a direct pathway regulator such as a protein phosphatase. Several targets of Skn7p that are required for the oxidative stress response have been identified (Morgan *et al.* 1997, Lee *et al.* 1999), however the genes that are regulated by Skn7p in response to cellular osmotic state are not yet known. By using a genome transcript profiling approach, the identity of all Skn7p targets may eventually be ascertained.

MID2 was isolated by J. Brown (personal communication) as a multicopy activator of Skn7p. Previously, Mid2p was found to be required for cell survival following exposure to mating pheromone (Ono *et al.* 1994). I found that Mid2p possessed properties that suggested that it might act as a sensor protein. Mid2p is localized uniformly throughout the plasma membrane, and contains a large, O-mannosylated extracellular domain that is essential for its function. Cell wall mutants with modified *MID2* gene dosage have altered growth rates and viability, *mid2Δ* mutants are resistant to the cell wall-binding drug calcofluor white, and cell wall chitin synthesis is altered in *mid2Δ* mutants and cells overexpressing *MID2*. These observations suggested that Mid2p is involved in cell wall synthesis. Searches for multicopy suppressors of various *mid2Δ* phenotypes isolated various components of the cell wall integrity pathway, and demonstrated that *MTL1* encodes a functional homolog of Mid2p. A number of genetic and biochemical approaches demonstrated that Mid2p is an upstream regulator of the *PKC1-MPK1* cell integrity pathway. Genetic interactions between *MID2* and genes that encode Rho1p GAP and GEF proteins, and the observation that high copy *MID2* increases Rho1p GEF activity suggests that Mid2p regulates the cell integrity pathway via modulation of Rho1p GEF activity. An intriguing problem that remains to be solved is how Mid2p senses cell wall state. Its O-mannosylated, extracellular domain is essential for activity. However this region is not

covalently bound to cell wall components. The extracellular domain is devoid of known ligand binding sequences, and is largely composed of non-complex, repetitive serine and threonine-rich sequence. One possibility is that Mid2p might act as a sensor of plasma membrane stretch. Plasma membrane stretch has been proposed as a trigger for *PKC1-MPK1* pathway activity (Kamada *et al.* 1995). Stretching of the plasma membrane could occur in regions of the cell where the cell wall is altered or damaged, and is insufficiently protecting the integrity of the plasma membrane. A second possibility is that Mid2p might act as a mechanosensor of cell wall state. The extracellular domain could non-covalently interact with either specific proteins or polysaccharides in the cell wall. Disruption or alteration of cell wall synthesis could conceivably interfere with this interaction, or physically alter Mid2p conformation, triggering Mid2p function.

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