Yeast Nap1-Binding Protein Nbp2p Is Required for Mitotic Growth at High Temperatures and for Cell Wall Integrity

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ABSTRACT

Nbp2p is a Nap1-binding protein in *Saccharomyces cerevisiae* identified by its interaction with Nap1 by a two-hybrid system. *NBP2* encodes a novel protein consisting of 236 amino acids with a Src homology 3 (SH3) domain. We showed that *NBP2* functions to promote mitotic cell growth at high temperatures and cell wall integrity. Loss of Nbp2 results in cell death at high temperatures and in sensitivity to calcofluor white. Cell death at high temperature is thought not to be due to a weakened cell wall. Additionally, we have isolated several type-2C serine threonine protein phosphatases (PTCs) as multicopy suppressors and MAP kinase-kinase (MAPKK), related to the yeast PKC MAPK pathway, as deletion suppressors of the *nbp2*Δ mutant. Screening for deletion suppressors is a new genetic approach to identify and characterize additional proteins in the Nbp2-dependent pathway. Genetic analyses suggested that Ptc1, which interacts with Nbp2 by the two-hybrid system, acts downstream of Nbp2 and that cells lacking the function of Nbp2 prefer to lose Mkk1, but the PKC MAPK pathway itself is indispensable when Nbp2 is deleted at high temperature.

NUCLEOSOME assembly protein 1 (Nap1) was identified in mammalian cell extracts by its intrinsic ability to facilitate nucleosome assembly *in vitro* in physiological ionic conditions (Ishimi *et al.* 1983). Its homologs, including TAF1/set proteins, are expressed in abundance in most eukaryotes (Nagata *et al.* 1995, 1998; Kawase *et al.* 1996; Matsumoto *et al.* 1999). Functional analyses, *in vitro*, have suggested that they are necessary to keep proper nucleosome structures in transcription and replication (Walter *et al.* 1995; Chang *et al.* 1997; Ito *et al.* 1997). In yeasts, additional functions have been ascribed to Nap1, as it has been shown to interact with Clb2 and Gin4, which are required for the proper control of mitotic events (Kellogg *et al.* 1995; Kellogg and Murray 1995; Altman and Kellogg 1997).

We first identified two genes (*NBP1*, *NBP2*) that encode proteins that interact with Nap1 by the two-hybrid system. *NBP1*, an essential gene with a coiled-coil structure in the center of the predicted amino acid sequence, encodes a protein localized in the nucleus as one or two tiny dots (Shimizu *et al.* 2000). On the other hand, *NBP2*, which contains a Src homology 3 (SH3) domain, encodes a novel protein consisting of 236 amino acids. SH3 domains constitute a family of protein-protein interaction modules that participate in diverse signaling pathways (*e.g.*, cell cycle control, signal transduction, or cytoskeleton organization). From the Yeast Genome Database, 25 gene products in *Saccharomyces cerevisiae* contain at least one copy of the SH3 domain (Mayer 2001). Nbp2 shares structural homology with a *Schizosaccharomyces pombe* protein Skb5 (36% overall identity), a direct activator of Shk1 kinase (Yang *et al.* 1999).

Mitogen-activated protein kinase (MAPK) cascades control changes in gene expression, cytoskeletal organization, and cell division (Herskowitz 1995; Levin and Errede 1995). In the PKC (protein kinase C) pathway, Pkc1 regulates a protein kinase cascade in which MAPKKK Bck1 activates the redundant MAPKKs, Mkk1 and Mkk2, which in turn activate MAPK Mpk1. Mutants that perturb signaling through this pathway display phenotypes indicative of a defect in cell wall integrity. The polymorphic locus *SSD1* is also important for promoting proper cell wall structure and integrity (Kaeberlein and Guarente 2002). Laboratory strains are divided into two types of *SSD1* alleles, *SSD1-V* and *ssd1-d*. *SSD1-V* alleles can suppress the lethality due to a deletion of *SIT4*, while *ssd1-d* alleles are synthetically lethal in combination with *sit4*Δ deletion mutants (Sutton *et al.* 1991). The *SSD1* gene functions on a separate branch of the PKC MAPK pathway (Kaeberlein and Guarente 2002).

In this report, we show that cells lacking *NBP2* exhibit cell death at high temperatures. In addition, *nbp2*Δ deletion mutants are sensitive to calcofluor white (CFW), indicating a defect in cell wall integrity. At high temperatures, *nbp2*Δ mutants failed to grow on medium supplemented with sorbitol, suggesting that *NBP2* is required for additional functions other than cell wall integrity. Fur-

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ther, we used a new genetic approach in yeast to identify and characterize additional proteins in the Nbp2-dependent pathway. We isolated five genes (*PTC1*, *PTC2*, *PTC4*, *MSB1*, *SKT5*) as multicopy suppressors and six genes (*GRE1*, *SEF1*, *MKK1*, *MKK2*, *YFR016C*, *PYK2*) as deletion suppressors. A recent article also identified *nbp2*Δ synthetic lethal/sick genes (Tong *et al.* 2001). These data lead us to propose an *NBP2* network (including *NAP1*), and we discuss a possible role of *NBP2* in the cellular signaling system.

MATERIALS AND METHODS

Strains, growth conditions, and transformations: Yeast strains used in this study are listed in Table 1. Wild-type strains W303 and YPH499 are known to carry the defective *SSD1* (C-terminal truncated) allele, called *ssd1-d* type (Ueson o *et al.* 1997). Yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose, 400 mg/liter of adenine) and SD (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with auxotrophic requirements. Yeast transformations were carried out by Frozen-EZ yeast transformation II (Zymo Research). *Escherichia coli* DH5α was used to propagate all plasmids. *E. coli* cells were cultured in Luria broth medium (1% tryptone, 0.5% yeast extract, 1% NaCl) and transformed by standard methods.

Construction of deletion mutants: All gene disruptions were constructed using the described plasmids (Table 2). To delete *NBP2*, the following plasmid was constructed. The 790-bp *Mun*I-*Hin*dIII fragment containing the 3′ noncoding region of the *NBP2* and a 506-bp *Eco*RI-*Hinc*II fragment containing the 5′ end were blunt-end ligated into the *Sal*I and *Kpn*I sites of pMB-7 (provided by Neil A. R. Gow; see Fonzi and Irwin 1993) to produce pKO110, which contains the *nbp2::hisG-URA3-hisG* allele. This plasmid was digested with *Sac*I and *Sph*I and transformed into the wild-type strains. Deletion of *NBP2* in Ura$^+$ transformants was confirmed by PCR. The *nbp2::hisG* allele was derived from the *nbp2::hisG-URA3-hisG* allele after selection on 1.0 mg/ml 5-fluoroorotic acid.

For construction of the *ptc1*Δ and *nap1*Δ alleles, the 0.7-kbp *Nde*I-*Ase*I fragment of *PTC1* and the 0.6-kbp *Rsa*I-*Xho*I fragment of *NAP1* were replaced with *LEU2*. For construction of the *mkk1*Δ and *nap1*Δ alleles, the 1.4-kbp *Bsr*GI-*Nde*I fragment of *MKK1* and the 0.7-kbp *Nsp*V-*Xho*I fragment of *NAP1* were replaced with *URA3*. For construction of the *ptc2*Δ, *ptc4*Δ, *mkk1*Δ, and *mkk2*Δ alleles, the 1.2-kbp *Xba*I-*Nco*I fragment of *PTC2*, the 0.3-kbp *Nru*I fragment of *PTC4*, the 1.0-kbp *Aat*II fragment of *MKK1*, and the 1.5-kbp *Nco*I fragment of *MKK2* were replaced with *HIS3*. For construction of the *bck1*Δ and *mpk1*Δ alleles, the 3.8-kbp *Bam*HI-*Sph*I fragment of *BCK1* and the 1.3-kbp *Hin*dIII fragment of *MPK1* were replaced with *TRP1*. Deletion mutants of components of the PKC MAPK pathway (*bck1*Δ, *mkk1*Δ, *mkk2*Δ, *mpk1*Δ) in W303 background were constructed on SD supplemented with 20% sorbitol. All of the deletion alleles were confirmed by PCR.

Plasmids: Plasmids used in this study are listed in Table 2. To examine the subcellular localization of Nbp2, green fluorescent protein (GFP) was fused to the carboxy terminus of Nbp2 and expressed from its own promoter in a multicopy plasmid. To produce Nbp2-GFP (YEUpNBP2-GFP), the 0.76-kbp *Nhe*I-*Hin*dIII fragment containing GFP was ligated into the *Nhe*I-*Hin*dIII gap of YEUpNBP2.

Screening for multicopy suppressors: The *nbp2*Δ cells (YPH499 nbp2::URA3: *MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 ssd1-d nbp2::URA3*) were transformed with a yeast genomic library (provided by Y. Ohya) constructed in the multicopy vector YEp13. After 3 days of growth on SD-Leu plates at 30°, Leu$^+$ transformants were transferred onto SD-Leu plates and colonies growing at 37° were obtained. Plasmids were isolated from the candidate transformants and transformed back into the *nbp2*Δ cells. A total of eight positive candidates were finally confirmed. The insert sequences in the candidate plasmids were sequenced from both ends to identify the region of the genome present. For those plasmids with more than one gene in the DNA insert, restriction fragments were subcloned to identify the responsible gene. DNA corresponding to the genes of interest was cloned into YEp13 or YEp51B and then transformed into various *nbp2*Δ cells.

Viability assay: Yeast cells were precultured on YPD plates at 26° for 2 days, yielding stationary cells. These cells were collected and washed with deionized water. Approximately 1×10^3 cells were spread on prewarmed (37°) YPD plates and cultured at 37°. After that, cells were shifted at 26° and live cells were counted after 3–5 days.

Screening for deletion suppressors: A mutagenized yeast genomic library was provided by Michael Snyder (see Burns *et al.* 1994). The mutagenized yeast DNA sequences were released from vector DNA by digestion with *Not*I and introduced into the *nbp2*Δ cells (YPH499 nbp2::URA3) by transformation and selection for the *LEU2* marker in the transposon. After 7 days growth on SD-Leu plates at 36°, growing cells were selected. A total of five positive candidates among 8000 Leu$^+$ transformants were finally confirmed. To determine the identity of sequences whose lacZ-Leu2-Amp fusion proteins localize to discrete sites within the cell, TaKaRa LA PCR *in vitro* cloning kit was used. The sequences fused to *lacZ* or *AMP* were determined using primers S2(lacZ) or S2(Amp(r)). Oligonucleotide primer sequences were as follows: S1(lacZ), 5′-AAAG CGCCATTCGCCATTCAGGCTG-3′; S2(lacZ), 5′-TTGGGTA ACGCCAGGGTTTTCCCAG-3′; S1(Amp(r)), 5′-AAGTTGCA GGACCACTTCTGCGCTC-3′; S2(Amp(r)), 5′-TTATCTACA CGACGGGGAGTCAGGC-3′.

Cell lysis assay: The cell lysis assay detecting extracellular alkaline phosphatase was described by Cabib and Duran (1975) and applied with the modifications described by Paravicini *et al.* (1992).

GeneChip analysis: Yeast strains used in GeneChip analysis were isogenic pairs of RAY (wild type) and YKO213 (*nbp2*Δ) or W303 (wild type) and YKO215 (*nbp2*Δ). Cells were precultured in YPD medium to an OD_{600} of 0.1 at 26°. The liquid culture was separated and then cells were grown for 4 hr at 26° or 37°. Total RNA preparation, poly(A)$^+$ RNA purification, and GeneChip analysis were carried out as described (Ohkuni *et al.* 2003). Oligonucleotide arrays (GeneChip Yeast Genome S98 Arrays) were manufactured by Affymetrix.

Fluorescence microscopy: Cells expressing GFP were grown in SD medium to an OD_{600} of 0.5–0.7. To stain nuclear DNA, cells were incubated with 10 μg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) for 15 min. Samples were examined with a fluorescence microscope (Olympus BX-60) using a ×100 UPlan Apo objective equipped with phase-contrast optics. GFP fluorescence was detected with filter XF104-2 (Omega Optical, Brattleboro, VT).

Two-hybrid assay: The strains and plasmids for two-hybrid analysis are listed in Tables 1 and 2, respectively. Plasmids pGAD-NBP1 and pGAD-NBP2 were isolated by two-hybrid screening. Plasmids pBTM-SIR4 and pGAD-SIR4 were used as a positive control. Yeast strains were grown to stationary phase in SD medium lacking leucine and tryptophan, diluted to 5×10^6 cells per milliliter, and then incubated at 30° for 3–4 hr. β-Galactosidase activity was determined as described by Vojtek *et al.* (1993).

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference or source
Derived from 4795-408		
4795-408	*MAT***a** *his7 leu2 ura3 ade2 ade3 can1 sap3*	L. Hartwell
YKO200	*MAT***a** *his7 leu2 ura3 ade2 ade3 can1 sap3 nbp2::hisG-URA3-hisG*	This work
YKO201	*MAT***a** *his7 leu2 ura3 ade2 ade3 can1 sap3 nbp2::hisG*	This work
Derived from RAY		
RAY	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$	Uesono *et al.* (1997)
YKO209	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG-URA3-hisG*	This work
YKO213	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG*	This work
YKO227	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nap1::LEU2*	This work
YKO232	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG mkk2::HIS3*	This work
YKO233	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG bck1::TRP1*	This work
YKO234	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG mkk1::HIS3*	This work
YKO235	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG mpk1::TRP1*	This work
YKO236	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *mpk1::TRP1*	This work
YKO237	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *mkk1::HIS3*	This work
YKO238	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *mkk2::HIS3*	This work
YKO240	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG nap1::URA3*	This work
YKO244	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG mkk1::URA3 mkk2::HIS3*	This work
YKO247	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *bck1::TRP1*	This work
YKO254	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *mkk1::URA3 mkk2::HIS3*	This work
YKO278	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *ptc2::HIS3*	This work
YKO279	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG ptc2::HIS3*	This work
YKO280	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *ptc4::HIS3*	This work
YKO281	*MAT***a** *ura3 leu2 trp1 his3 GAL*$^+$ *nbp2::hisG ptc4::HIS3*	This work
Derived from KA31		
KA31	*MAT***a** *ura3 leu2 trp1 his3*	Irie *et al.* (1993)
YKO210	*MAT***a** *ura3 leu2 trp1 his3 nbp2::hisG-URA3-hisG*	This work
YKO214	*MAT***a** *ura3 leu2 trp1 his3 nbp2::hisG*	This work
YKO222	*MAT***a** *ura3 leu2 trp1 his3 nbp2::hisG ptc1::LEU2*	This work
YKO226	*MAT***a** *ura3 leu2 trp1 his3 ptc1::LEU2*	This work
Derived from W303		
W303	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d*	Sutton *et al.* (1991)
YKO211	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG-URA3-hisG*	This work
YKO215	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG*	This work
YKO252	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d mpk1::TRP1*	This work
YKO253	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG mpk1::TRP1*	This work
YKO262	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG mkk2::HIS3*	This work
YKO264	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG mkk1::HIS3*	This work
YKO266	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG mkk1::URA3 mkk2::HIS3*	This work
YKO268	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1-100 ssd1-d nbp2::hisG bck1::TRP1*	This work
Derived from YPH499		
YPH499	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 ssd1-d*	Sikorski and Hieter (1989)
YKO212	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 ssd1-d nbp2::hisG-URA3-hisG*	This work
YKO216	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 ssd1-d nbp2::hisG*	This work
Two-hybrid system host		
L40	*MAT***a** *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*	Vojtek *et al.* (1993)

RESULTS

***NBP2* is essential at high temperatures:** To characterize the function of *NBP2*, we introduced a disruption of *NBP2* (*nbp2*Δ) in several yeast strains of different backgrounds. Deletion alleles of *NBP2* (*nbp2::hisG-URA3-hisG*) were constructed as described in materials and methods. In five representative strains, the *nbp2*Δ mutants failed to grow at high temperatures (Table 3 and Figure 1A). These cells, once incubated at high temperatures for 2 days, could not recover their growth again at a room temperature (Table 3), suggesting that *nbp2*Δ cells are

TABLE 2

Plasmids used in this study

Plasmid	Vector	Description	Reference or source
Multicopy or single-copy plasmids			
YEp13		2μ *LEU2*	Parent *et al.* (1985)
YEp51B		2μ *LEU2*	Ohkuni and Yamashita (2000)
YEp24		2μ *URA3*	Botstein *et al.* (1979)
YEUp3		2μ *URA3*	Uesono *et al.* (1994)
pRS415		*ARS/CEN6 LEU2*	Stratagene (La Jolla, CA)
YCp19		*ARS/CEN4 LEU2 URA3*	K. Tanaka
pKO100	YEp51B	2μ *LEU2 PTC2*	This work
pKO102	YEp51B	2μ *LEU2 PTC4*	This work
pKO104	YEp13	2μ *LEU2 MSB1*	This work
pKO105	YEp51B	2μ *LEU2 SKT5*	This work
pKO116	YEp24	2μ *URA3 PTC1*	This work
pKO117	YEp51B	2μ *LEU2 PTC1*	This work
YEUpNBP2	YEUp3	2μ *URA3 NBP2*	This work
pRSL-NBP2	pRS415	*ARS/CEN6 LEU2 NBP2*	This work
pYK915	YCp19	*ARS/CEN4 LEU2 SSD1*	Y. Kikuchi
Deletion plasmids			
pKO110		*nbp2::hisG-URA3-hisG* deletion plasmid	This work
pDPD111		*ptc1::LEU2* deletion plasmid	T. Maeda
pKO112		*ptc2::HIS3* deletion plasmid	This work
pKO113		*ptc4::HIS3* deletion plasmid	This work
pΔbck1::TRP1		*bck1::TRP1* deletion plasmid	Y. Kikuchi
pKO118		*mkk1::HIS3* deletion plasmid	This work
pKO129		*mkk2::HIS3* deletion plasmid	This work
pKO137		*mkk1::URA3* deletion plasmid	This work
pΔmpk1::TRP1		*mpk1::TRP1* deletion plasmid	Y. Kikuchi
pTN5		*nap1::LEU2* deletion plasmid	This work
pKO127		*nap1::URA3* deletion plasmid	This work
GFP fusion plasmid			
YEUpNBP2-GFP	YEUpNBP2	2μ *URA3 NBP2-GFP*	This work
Two-hybrid system			
pBTM116		2μ P_{ADH}-*lexA TRP1*	Shimizu *et al.* (2000)
pGAD		2μ P_{ADH}-*GAL4 ad LEU2*	Chien *et al.* (1991)
pBTM-NAP1	pBTM116	2μ P_{ADH}-*lexA-NAP1*$_{(1-417)}$ *TRP1*	Shimizu *et al.* (2000)
pBTM-SIR4	pBTM116	2μ P_{ADH}-*lexA-SIR4 TRP1*	R. Sternglanz
pGAD-NBP1	pGAD	2μ P_{ADH}-*GAL4 ad-NBP1*$_{(34-319)}$ *LEU2*	This work
pGAD-NBP2	pGAD	2μ P_{ADH}-*GAL4 ad-NBP2*$_{(155-236)}$ *LEU2*	This work
pGAD-SIR4	pGAD	2μ P_{ADH}-*GAL4 ad-SIR4 LEU2*	R. Sternglanz

killed at restrictive temperatures. We therefore tested cell viability at 37° of *nbp2*Δ mutants, and a significant level of cell death was observed at 37° in the *nbp2*Δ cells (Figure 1B). These results indicate that *NBP2* is essential for mitotic growth at high temperatures.

Isolation of multicopy suppressors of the *nbp2*Δ: To identify additional components of Nbp2-dependent cell viability at high temperature, we isolated genes that, when overexpressed from multicopy plasmids, are capable of suppressing the growth defect of *nbp2*Δ mutants (YPH499 in regard to *nbp2*Δ) at high temperatures. Eight candidate genes cloned in the multicopy vector YEp13 were obtained. Sequence analysis of subclones containing the suppressor activity revealed that these DNA fragments are identical in sequence to *PTC2*, *PTC4*, *MSB1*, and *SKT5*. Next, we reexamined these suppressors in a wild-type *SSD1* background (YKO201; 4795-408 in regard to *nbp2*Δ). In this background, *SKT5* could not suppress the *nbp2*Δ mutants (Figure 2). The wild-type strains W303 and YPH499 are known to carry defective *SSD1* alleles (Uesono *et al.* 1997). In fact, a single-copy of *SSD1* partially suppressed the temperature sensitivity of the *nbp2*Δ mutation in the W303 or YPH499 backgrounds (Table 4). This result suggests that *SKT5* may be a multicopy suppressor of the *ssd1* mutation.

PTC2 and *PTC4* encode proteins belonging to the type-2C serine threonine protein phosphatase (PP2C) class of highly conserved protein family found in all

TABLE 3

Temperature sensitivity of the *nbp2*Δ strains

Strain	Relevant genotype[a]	Growth on YPD[b] 37°	(RG)	38°	(RG)	CFW sensitivity[c]
4795-408	*NBP2 SSD1*	+		−	−	R
YKO201	*nbp2*Δ *SSD1*	−	−	−	−	S
RAY	*NBP2 SSD1*	+		−	+	R
YKO209	*nbp2*Δ *SSD1*	−	−	−	−	S
KA31	*NBP2 SSD1*	+		+		R
YKO210	*nbp2*Δ *SSD1*	−	+	−	−	S
W303	*NBP2 ssd1-d*	+		−	+	R
YKO211	*nbp2*Δ *ssd1-d*	−	+	−	−	S
YPH499	*NBP2 ssd1-d*	+		−	−	R
YKO212	*nbp2*Δ *ssd1-d*	−	−	−	−	S

[a] *SSD1* in the strains W303 and YPH499 encodes a C-terminally truncated protein (*ssd1-d*; Uesono *et al.* 1997).

[b] Cells were streaked onto YPD medium and incubated for 2 days at 37° or 38°: +, growth; −, no growth. (RG) indicates growth when cells were returned to room temperature (26°) after the high temperature treatment: +, recovery growth; −, cell death.

[c] R indicates that the given strain is resistant and grew well and S indicates that the cells are sensitive and grew poorly on YPD solid medium supplemented with 0.1 mg/ml CFW.

eukaryotes (Cheng *et al.* 1999). Since *PTC1* encoding a type-2C serine threonine protein phosphatase (Jiang *et al.* 1995) was shown to interact with Nbp2 in a comprehensive two-hybrid assay (Ito *et al.* 2000; Uetz *et al.* 2000), we tested whether a multicopy plasmid carrying *PTC1* suppresses *nbp2*Δ. *PTC1* was found to suppress

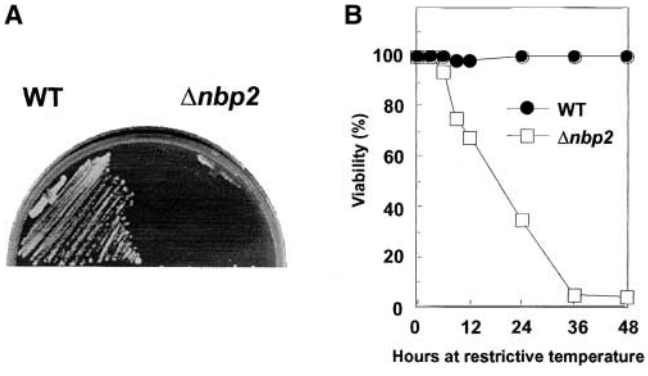


Figure 1.—*nbp2*Δ cells were observed for cell death at restrictive temperatures. Isogenic yeast strains 4795-408 (wild type) and YKO201 (*nbp2*Δ) were cultured. (A) Yeast cells were streaked onto YPD medium and incubated at 37° for 2 days. (B) Yeast cells were precultured on YPD medium at 26° for 2 days and then dilutions of cells plated onto YPD medium were shifted to 37° for the indicated times. After growth at 26° for 3–5 days, living cells were determined. WT, wild type.

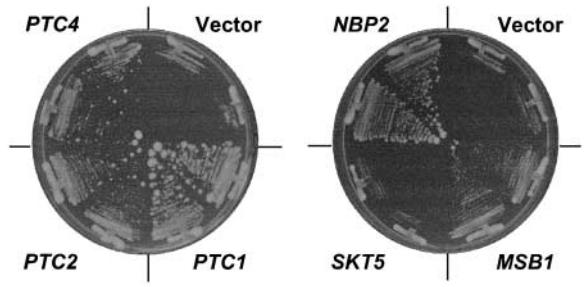


Figure 2.—Multicopy suppressors of the temperature-sensitive growth of the *nbp2*Δ mutant. The *nbp2*Δ mutant, YKO201, transformed with different plasmids, was streaked onto YPD medium and incubated at 36° for 3 days. Plasmids are YEp51B (Vector), pKO117 (*PTC1*), pKO100 (*PTC2*), pKO102 (*PTC4*), pKO104 (*MSB1*), pKO105 (*SKT5*), and RSL-NBP2 (*NBP2*).

a temperature-sensitive *nbp2*Δ allele (Figure 2). These results suggest that these PP2C phosphatases may be important in the control of mitotic growth at high temperatures in *nbp2*Δ cells.

To examine genetic interaction between *NBP2* and *PTC*s, single deletion mutants of *PTC*s (*ptc1*Δ, *ptc2*Δ, and *ptc4*Δ) and double deletion mutants (*nbp2*Δ *ptc1*Δ, *nbp2*Δ *ptc2*Δ, and *nbp2*Δ *ptc4*Δ) were constructed. Three mutants, *nbp2*Δ, *ptc1*Δ, and *nbp2*Δ *ptc1*Δ, were normal in their growth on YPD at 26° but failed to grow at an elevated temperature (Figure 3A). When transferred to a high temperature, *nbp2*Δ gradually lost its viability, while the *nbp2*Δ *ptc1*Δ double mutant did so more rapidly—at the same rate as the *ptc1*Δ mutant (Figure 3B). We also tested the ability of a multicopy plasmid carrying *NBP2* to complement the growth defect of the *ptc1*Δ cells at 37°. *NBP2* could not suppress a temperature-sensitive *ptc1*Δ allele (Figure 3C). These results suggest that *PTC1* is also essential at high temperatures and that *PTC1* acts downstream of *NBP2*.

On the other hand, two single mutants, *ptc2*Δ or *ptc4*Δ, were grown normally on YPD at 26° or 37°, but the double mutants *nbp2*Δ *ptc2*Δ or *nbp2*Δ *ptc4*Δ failed to grow at an elevated temperature (Figure 3D). These double mutants lost their viability more rapidly than the *nbp2*Δ mutant did (Figure 3E), suggesting that genetic interactions between *NBP2* and *PTC2* or *PTC4* are parallel pathways to promote mitotic growth at high temperatures.

Isolation of deletion suppressors of the *nbp2*Δ: To search for genes involved in the function of *NBP2*, we tried to identify genes that could suppress the growth defect of the *nbp2*Δ mutants at high temperatures when deleted (see materials and methods). A total of 8000 Leu^+ transformants, carrying random deletions in addition to *nbp2*Δ, were obtained and subsequently screened for their ability to grow at the restrictive temperature of 36°. Five transformants were capable of forming colonies (Figure 4). Sequence analysis (see materials and methods) revealed that these five disruptants inacti-

TABLE 4

Synthetic sick interactions between *NBP2* and *SSD1*

Strain	Relevant genotype	36°			37°			38°		
		Vector	*NBP2*	*SSD1*	Vector	*NBP2*	*SSD1*	Vector	*NBP2*	*SSD1*
4795-408	*NBP2 SSD1*	+	+	+	+	+	+	−	−	−
YKO201	*nbp2*Δ *SSD1*	−	+	−	−	+	−	−	−	−
RAY	*NBP2 SSD1*	+	+	+	+	+	+	−	−	−
YKO213	*nbp2*Δ *SSD1*	−	+	−	−	+	−	−	−	−
W303	*NBP2 ssd1-d*	+	+	+	+	+	+	−	−	+
YKO215	*nbp2*Δ *ssd1-d*	−	+	+	−	+	−	−	−	−
YPH499	*NBP2 ssd1-d*	+	+	+	−	−	+	ND	ND	ND
YKO216	*nbp2*Δ *ssd1-d*	−	+	+	ND	ND	ND	ND	ND	ND

Growth of the indicated strains, transformed with different single-copy plasmids, was measured by visual observation of cells incubated on YPD or SD plates for 2 or 3 days. Plasmids are pRS415 (Vector), pRSL-NBP2 (*NBP2*), and pYK915 (*SSD1*). +, grew well; −, grew very slowly or had no observable growth; ND, the experiment was not performed.

vated one of the following genes: *SEF1* (protein with similarity to transcription factors; Poch 1997), *MKK1* (MAPKK; Irie *et al.* 1993), *PYK2* (pyruvate kinase; Boles *et al.* 1997), and the promoter regions of *GRE1* (insertion at 14 bp upstream of the initiation codon; unknown function) and *YFR016C* (insertion at 470 bp upstream of initiation codon; unknown function). We also tested using another yeast background (KA31; wild-type *SSD1*). *nbp2*Δ *pyk2*Δ and *nbp2*Δ *yfr016c*Δ double disruptants in the KA31 background could not suppress the growth defect of *nbp2*Δ cells (data not shown). These results indicate that *PYK2* and *YFR016C* may behave as deletion suppressors of the *ssd1* mutation.

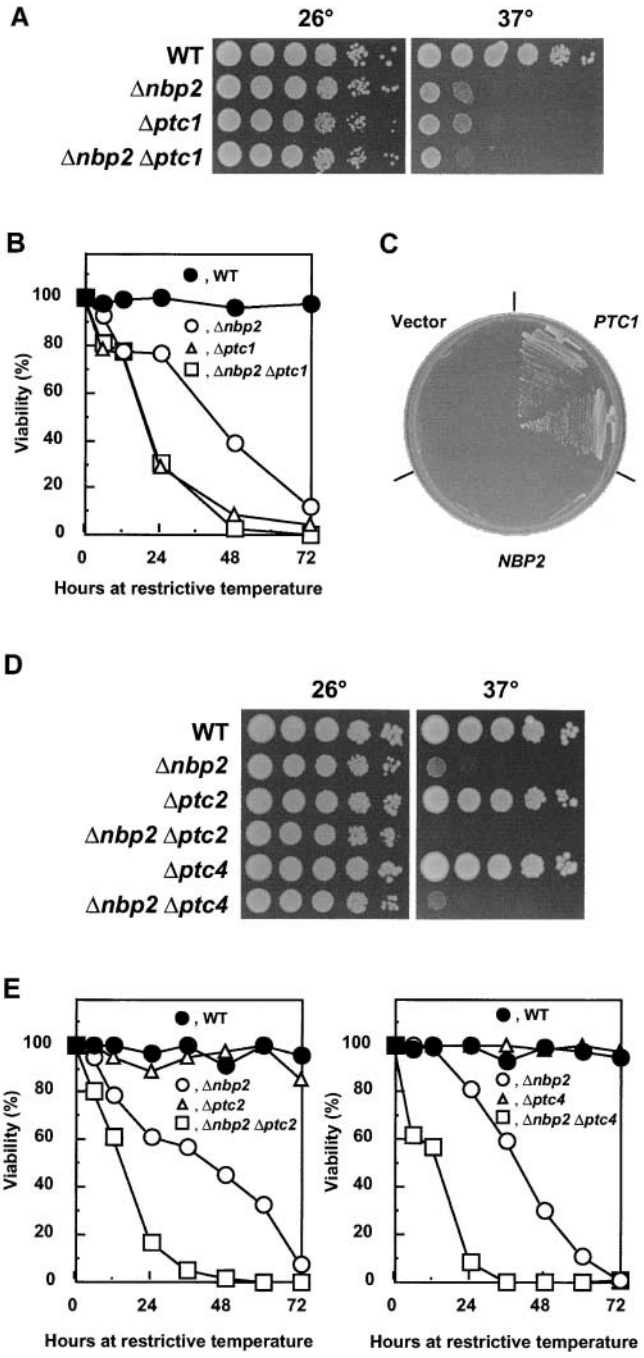
***MKK* has a negative effect on *NBP2* function at high temperature:** To investigate genetic interactions between *NBP2* and components of the PKC MAPK pathway, five deletion mutants, *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ, *nbp2*Δ *mkk2*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ were constructed in two backgrounds. We first examined the temperature sensitivity of *nbp2*Δ cells in a wild-type *SSD1* background (Figure 5A). The *nbp2*Δ *mkk1*Δ and *nbp2*Δ *mkk2*Δ double disruptants could partially suppress the growth defect of *nbp2*Δ cells on YPD solid medium at a high temperature. The *MKK1* and *MKK2* genes are functionally redundant and are 31% identical at the N-terminal half and 80% identical at the C-terminal half (Irie *et al.* 1993). In contrast, growth of the disruptants, *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ, on YPD solid medium at high temperatures was more severely affected than that of the *nbp2*Δ cells. Wild-type cells could grow at elevated temperatures by addition of 20% sorbitol. The disruptants, *nbp2*Δ, *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ, could not grow on YPD supplemented with 20% sorbitol at 38°, while the *bck1*Δ and *mpk1*Δ single disruptants could. These results suggest that the loss of either *MKK1* or *MKK2* alleviates loss of the Nbp2 function at high temperatures, although the PKC MAPK pathway itself is indispensable for cell growth.

We next examined the temperature sensitivity of the *nbp2*Δ cells in defective *SSD1* alleles (Figure 5B). In this background, the *nbp2*Δ *mkk1*Δ and *nbp2*Δ *mkk2*Δ double disruptants could partially suppress the growth defect of the *nbp2*Δ cells on YPD solid medium at high temperatures (data not shown). On the other hand, the *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ disruptants could no longer grow on YPD solid medium at 26°. These disruptants could grow on YPD supplemented with 20% sorbitol, indicating that they are deficient in cell wall integrity. Thus, Nbp2 may play a role in promoting cell wall stability.

***NBP2* also regulates cell wall integrity:** Mutations that perturb signaling through the PKC MAPK pathway can result in sensitivity to changes in external osmolarity, defective budding, and cell lysis (Levin and Errede 1995; Kaeberlein and Guarente 2002). To test a cell wall defect in *nbp2*Δ mutants, we first examined sensitivity to CFW, a cell wall perturbing agent. The *nbp2*Δ mutants were more sensitive to CFW than were wild-type cells (Table 3 and Figure 6A). Cell lysis was next examined on the basis of the extracellular release of alkaline phosphatase (Figure 6C). Two background *nbp2*Δ mutants were grown on YPD plates at 26° and incubated overnight at 37°, and then cell lysis assays were performed. The *mpk1*Δ mutant in a *ssd1-d* background turned dark blue after cell lysis assay, indicating that the cells lyse rapidly, while wildtype remain white. On the other hand, the *mpk1*Δ in a *SSD1-V* background and the *nbp2*Δ in a *ssd1-d* background turned pale blue, indicating that the cells lyse slowly. The PKC MAPK pathway and Ssd1 are

defined as parallel to regulate cell wall integrity (Kaeberlein and Guarente 2002). Thus, Nbp2 may influence cell wall integrity, but this effect is smaller than that of the PKC MAPK pathway.

We also examined the relationships between cell death at high temperatures and cell wall integrity. Interestingly, the sensitivity to CFW at 26° caused by the loss of Nbp2 is completely suppressed by addition of 20% sorbitol to the medium (Figure 6A). At a high temperature (38°), however, the *nbp2*Δ mutant failed to grow on YPD supplemented with CFW and 20% sorbitol, suggesting that *NBP2* at high temperatures is required for additional functions other than cell wall integrity.



We further examined the CFW sensitivity of the *nbp2*Δ cells related to the PKC MAPK pathway (Figure 6B). All of the disruptants except for *mkk1*Δ and *mkk2*Δ single mutants were sensitive to CFW. The CFW sensitivity of these disruptants except for the *nbp2*Δ *mkk2*Δ double mutant could be suppressed by addition of 20% sorbitol to the medium, indicating that *NBP2* and components of the PKC MAPK pathway play similar roles in promoting cell wall stability. On the other hand, the CFW sensitivity of the *nbp2*Δ *mkk2*Δ double disruptant could be partially suppressed by loss of Mkk1. These observations indicate that cells lacking the function of Nbp2 grow better in the absence of *MKK1* and *MKK2* for maintaining cell wall integrity.

GeneChip analysis: It is possible that the suppression of temperature-sensitive growth in *nbp2*Δ cells was the consequence of gene expression of *PTC*s and the PKC MAPK pathway. To test this, we measured the mRNA levels of *PTC*s (*PTC1*, *PTC2*, *PTC4*) and PKC MAPK pathway genes (*BCK1*, *MKK1*, *MKK2*, *MPK1*) in wild-type and *nbp2*Δ cells in the cells with two genetical backgrounds (*SSD1-V* and *ssd1-d*) at two growth temperatures, 26° or 37°, using the GeneChip method. In either background, by shifting growth temperature from 26° to 37°, no significant differences (within twofold up- or downregulation) in mRNA levels of those genes were observed (data not shown).

Nbp2 localizes in the cytoplasm: To investigate the subcellular localization of Nbp2, we constructed a fusion protein with GFP. GFP was fused to a C terminus of the Nbp2 and expressed from a multicopy plasmid in a *nbp2*Δ strain. This multicopy Nbp2-GFP plasmid complemented the temperature sensitivity of the *nbp2*Δ strain at 37° (data not shown). In most cells, Nbp2-GFP

Figure 3.—Genetic interactions between *NBP2* and *PTC*s. Isogenic yeast strains used for A–C were as follows: KA31 (wild type), YKO214 (*nbp2*Δ), YKO226 (*ptc1*Δ), and YKO222 (*nbp2*Δ *ptc1*Δ). (A) Yeast cells grown for 2 days on YPD medium were harvested, washed, normalized by OD_{600}, and spotted onto YPD plates in a series of six 10-fold dilutions. Plates were allowed to grow for 3 days at 26° or 37°. (B) Yeast cells were precultured on YPD medium at 26° for 2 days and then dilutions of cells plated onto YPD medium were shifted to 37° for the indicated times. After growth at 26° for 3–5 days, living cells were determined. (C) The *ptc1*Δ mutant, YKO226, transformed with different plasmids, was streaked onto SD medium and incubated at 36.5° for 3 days. Plasmids are YEp24 (Vector), pKO116 (*PTC1*), and YEUpNBP2. Isogenic yeast strains used for D and E were as follows: RAY (wild type), YKO213 (*nbp2*Δ), YKO278 (*ptc2*Δ), YKO279 (*nbp2*Δ *ptc2*Δ), YKO280 (*ptc4*Δ), and YKO281 (*nbp2*Δ *ptc4*Δ). (D) Yeast cells grown for 2 days on YPD medium were harvested, washed, normalized by OD_{600}, and spotted onto YPD plates in a series of five 10-fold dilutions. Plates were allowed to grow for 3 days at 26° or 37°. (E) Yeast cells were precultured on YPD medium at 26° for 2 days and then dilutions of cells plated onto YPD medium were shifted to 37° for the indicated times. After growth at 26° for 3–5 days, living cells were determined. WT, wild type.

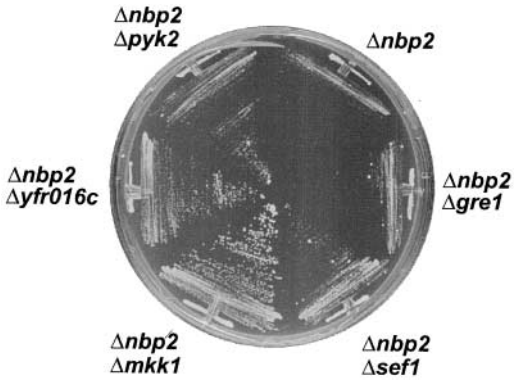


FIGURE 4.—Deletion suppressors of the temperature-sensitive growth of the *nbp2*Δ mutant. Deletion suppressors obtained were streaked onto SD medium and incubated at 35.5° for 5 days.

was found in cytoplasm; in some cells, several small spots were also observed in the cytoplasm (Figure 7). An experiment to determine the location of these small spots is in progress. Thus, the localization of Nbp2 is in the cytoplasm of a cell, suggesting that Nbp2 may play a role in a cellular signaling system in cytoplasm.

Synthetic sick interaction between Nbp2 and Nap1: In a previous article (SHIMIZU *et al.* 2000), we isolated Nbp2 that interacts with Nap1 by the two-hybrid system (Figure 8A). Here, we tried to determine the genetic interaction between *NBP2* and *NAP1* (Figure 8B). Cells lacking Nap1 could grow well at 37° and on YPD solid medium supplemented with 0.1 mg/ml CFW (data not shown). When tested for various phenotypes characteristic of the *nbp2*Δ *nap1*Δ double mutant, this disruptant was more sensitive at high temperatures and to CFW than were either *nbp2*Δ or *nap1*Δ mutants. But the *nbp2*Δ *nap1*Δ double mutant was slightly temperature sensitive compared with the *nbp2*Δ mutant, and new morphological defects at high temperatures were not observed (data not shown). On the other hand, the CFW sensitivity of the *nbp2*Δ *nap1*Δ double mutant could not be suppressed by addition of 20% sorbitol to the medium, indicating that this mutant may produce another defect in cellular integrity. Thus, these observations demonstrate that the relation between Nbp2 and Nap1 is a synthetic sick-type interaction.

DISCUSSION

Nap1 is necessary to keep proper nucleosome structures *in vitro* (WALTER *et al.* 1995; CHANG *et al.* 1997; ITO *et al.* 1997) and is required for the mitotic cyclin Clb2 to be able to execute a subset of its normal mitotic activities (KELLOGG and MURRAY 1995). Yet little is known about Nap1 *in vivo*, although in an earlier report we indicated that Nbp2, containing an SH3 domain, interacts with Nap1 by a two-hybrid system (SHIMIZU *et al.* 2000). In the present study, we demonstrate that the Nbp2 regulates mitotic cell growth at high temperatures and cell wall integrity. Furthermore, we have used a new genetic approach to identify the function of Nbp2. These genetic analyses suggest that Ptc pathways regulate cell death at high temperatures and that the PKC MAPK pathway regulates cell wall integrity together with Nbp2.

***nbp2*Δ cells have severe growth defects at high temperatures:** In *nbp2*Δ strains, cells could not grow at high temperatures and rapid loss of viability at a restrictive temperature was observed. This temperature sensitivity could not be suppressed by the addition of 20% sorbitol to the medium (Figure 6A), indicating that the major cause of cell death at high temperatures may reside in a process other than cell wall integrity. Cell death of *nbp2*Δ at high temperatures is regulated by several PP2Cs of the protein phosphatase class of enzymes. Genetic analyses suggest that *PTC1* acts downstream of *NBP2* and that both *PTC2* and *PTC4* act parallel to *NBP2*. *PTC1*, *PTC2*, and *PTC3* are known as negative regulators of the HOG (high-osmolarity glycerol) MAPK pathway (WARMKA *et al.* 2001). We isolated MAPKK, related to the yeast PKC MAPK pathway, as deletion suppressors of the *nbp2*Δ mutant. Therefore, it is possible that two MAPK pathways, PKC and HOG, cross-talk through *NBP2*.

A role for Nbp2 in promoting cell wall integrity: In all the *nbp2*Δ strains used in this study, cells could not grow on YPD supplemented with CFW, a cell wall perturbing agent, suggesting that *nbp2*Δ mutants have a primary defect in cell wall integrity. The strains we used can be divided into two groups, *SSD1-V* (active allele; 4795-408, RAY, KA31) and *ssd1-d* (inactive allele; W303, YPH499). *SSD1* can act in parallel to the PKC MAPK pathway to promote cell wall integrity (KAEBERLEIN and GUARENTE 2002). For several reasons the genetic interactions between *NBP2* and *SSD1* and the PKC MAPK pathway are parallel to promote cell wall integrity. First, the *NBP2* and *SSD1* pathway generates a parallel signal at high temperatures. As in the case of the *nbp2*Δ mutants in W303 and YPH499 backgrounds, a single-copy vector carrying *SSD1* could partially suppress the temperature-sensitive phenotype of the *nbp2*Δ mutant (Table 4). The *NBP2*, *ssd1-d* strains (W303, YPH499), transformed with the single-copy vector carrying *SSD1*, could grow to some extent at an elevated temperature (Table 4). These results suggest that the relation between *NBP2* and *SSD1* exhibits a synthetic sick phenotype. Second, the double disruptants, *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ were more sensitive than the single disruptants (Figure 5A), indicating that the relation between *NBP2* and the PKC MAPK pathway must be a parallel pathway. Third, the disruptants in a W303 background, *nbp2*Δ *bck1*Δ, *nbp2*Δ *mkk1*Δ *mkk2*Δ, and *nbp2*Δ *mpk1*Δ, could not grow on YPD at 26°, while they could grow on YPD supplemented with 20% sorbitol (Figure 5B), indicating that the synthetic lethality of these disruptants may come from a defect in cell wall stability.

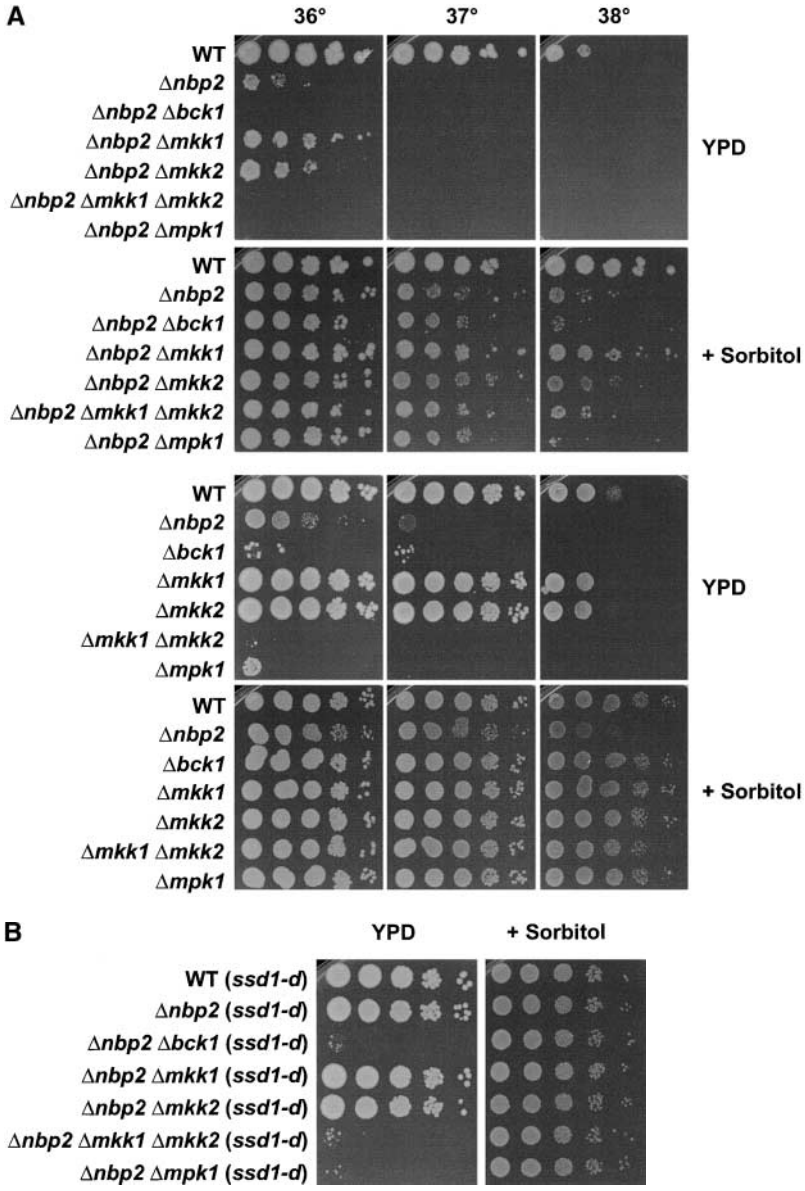


FIGURE 5.—Genetic interaction between *NBP2* and components of PKC MAPK pathway. Yeast cells grown for 2 days on YPD medium were harvested, washed, normalized by OD_{600}, and spotted onto the designated media in a series of five 10-fold dilutions. Plates were allowed to grow for 3 days. (A) Cells were cultured on YPD and YPD supplemented with 20% sorbitol at 36°–38°. Isogenic yeast strains in a *SSD1-V* background were as follows: RAY (wild type), YKO213 (*nbp2*Δ), YKO233 (*nbp2*Δ *bck1*Δ), YKO234 (*nbp2*Δ *mkk1*Δ), YKO232 (*nbp2*Δ *mkk2*Δ), YKO244 (*nbp2*Δ *mkk1*Δ *mkk2*Δ), YKO235 (*nbp2*Δ *mpk1*Δ), YKO247 (*bck1*Δ), YKO237 (*mkk1*Δ), YKO238 (*mkk2*Δ), YKO254 (*mkk1*Δ *mkk2*Δ), and YKO236 (*mpk1*Δ). (B) Cells were cultured on YPD and YPD supplemented with 20% sorbitol at 26°. Isogenic yeast strains in *ssd1-d* background were as follows: W303 (wild type), YKO215 (*nbp2*Δ), YKO268 (*nbp2*Δ *bck1*Δ), YKO264 (*nbp2*Δ *mkk1*Δ), YKO262 (*nbp2*Δ *mkk2*Δ), YKO266 (*nbp2*Δ *mkk1*Δ *mkk2*Δ), and YKO253 (*nbp2*Δ *mpk1*Δ). WT, wild type.

Finally, double-mutant cells carrying *mpk1*Δ, *nbp2*Δ, and *ssd1-d* were lysed more rapidly than single mutant cells (Figure 6C). Taken together, we believe that the *NBP2*, *SSD1*, and PKC MAPK pathways define three parallel pathways that regulate cell wall integrity.

We also suggest different roles for *MKK1* and *MKK2*, which correspond to MEK in *S. cerevisiae*, in the cell signaling system under the influence of *NBP2*. The CFW sensitivity of the *nbp2*Δ *mkk2*Δ double mutant is more severe than that of Δ*nbp2* Δ*mkk1* cells. The CFW sensitivity of the *nbp2*Δ *mkk2*Δ double mutant could not be suppressed by addition of 20% sorbitol to the medium, but could be suppressed by the loss of *MKK1* (Figure 6B). Thus, we propose that *MKK1* and *MKK2* are not necessarily redundant and that each gene may play a separate role in promoting cell wall integrity.

We have isolated *mkk1*Δ and *mkk2*Δ and loss of function of either *MKK1* or *MKK2* as deletion suppressors of *nbp2*Δ cells. But the PKC MAPK pathway itself is indispensable for cell wall maintenance in the *nbp2*Δ mutant. According to our GeneChip analysis, no significant differences in the mRNA amounts of components of the PKC MAPK pathway were observed between wild-type and *nbp2*Δ cells (data not shown). These results suggest that *MKK1* and *MKK2* may have roles other than in cell wall integrity in the Nbp2-dependent pathway.

A new genetic approach of deletion suppressor: In this study, we have used a novel genetic approach, which we have called deletion suppressor, to pursue characterization of Nbp2. We isolated six candidate genes as deletion suppressors of *nbp2*Δ cells. Until now, the isolation of multicopy suppressor and synthetic lethal alleles has been practiced as a standard method. By isolating a deletion suppressor, we can argue for, for example, a balance of two opposite pathways, such as kinase and phosphatase. As in Figure 9A, we think that if a gene

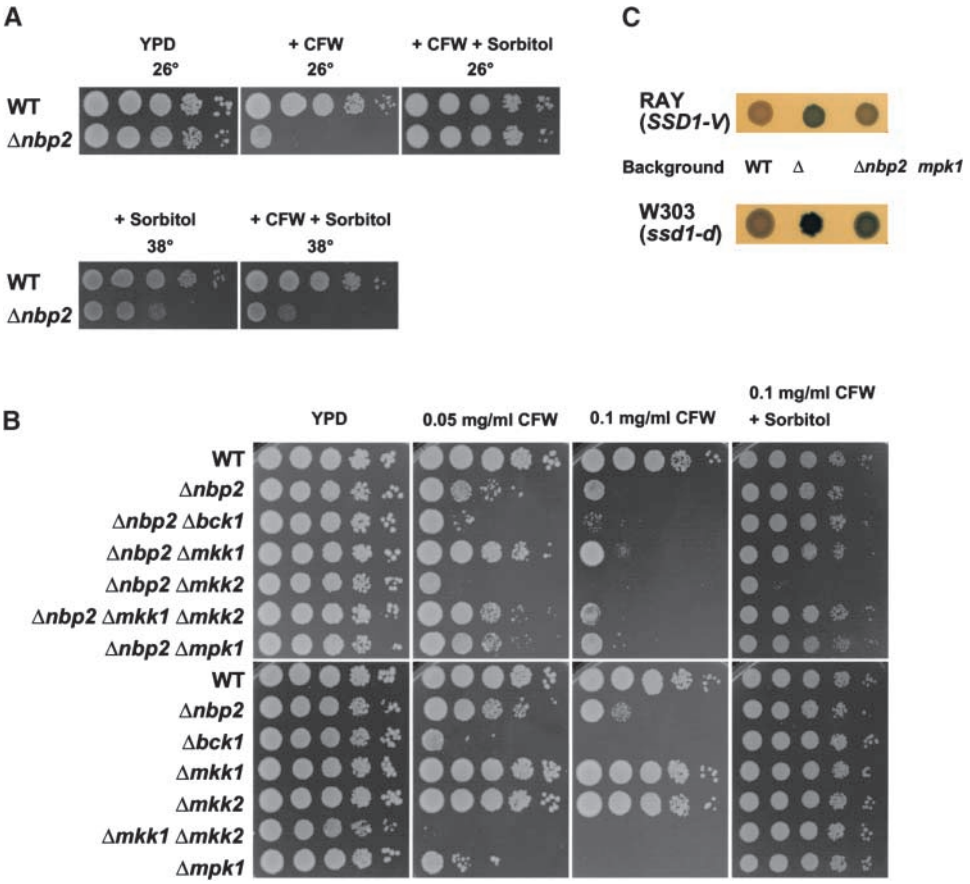


FIGURE 6.—Mutation of *NBP2* results in cell wall integrity. (A and B) Yeast cells grown for 2 days on YPD medium were harvested, washed, normalized by OD_{600}, and spotted onto the designated media in a series of five 10-fold dilutions. Plates were allowed to grow for 3 days. (A) Media in each plate were YPD, YPD supplemented with 0.1 mg/ml CFW (+CFW), YPD supplemented with 20% sorbitol (+Sorbitol), and YPD supplemented with 0.1 mg/ml CFW and 20% sorbitol (+CFW+Sorbitol). Isogenic yeast strains RAY (wild type) and YKO213 (*nbp2*Δ) were used. (B) Cells were cultured on YPD, YPD supplemented with 0.05 or 0.1 mg/ml CFW, and YPD supplemented with 0.1 mg/ml CFW and 20% sorbitol at 26°. Isogenic yeast strains used were as follows: RAY (wild type), YKO213 (*nbp2*Δ), YKO233 (*nbp2*Δ *bck1*Δ), YKO234 (*nbp2*Δ *mkk1*Δ), YKO232 (*nbp2*Δ *mkk2*Δ), YKO244 (*nbp2*Δ *mkk1*Δ *mkk2*Δ), YKO235 (*nbp2*Δ *mpk1*Δ), YKO247 (*bck1*Δ), YKO237 (*mkk1*Δ), YKO238 (*mkk2*Δ), YKO254 (*mkk1*Δ *mkk2*Δ), and YKO236 (*mpk1*Δ). (C) Cell lysis assay of the *nbp2*Δ cells. Approximately 10^5 cells were spotted onto YPD plates, cultivated for 2 days at 26°, and then incubated overnight at 37°. The plates were then overlaid with an alkaline phosphatase assay solution. Isogenic yeast strains in a *SSD1-V* background were as follows: RAY (wild type), YKO236 (*mpk1*Δ), and YKO213 (*nbp2*Δ). Isogenic yeast strains in a *ssd1-d* background were as follows: W303 (wild type), YKO252 (*mpk1*Δ) and YKO215 (*nbp2*Δ). WT, wild type.

responsible for negative effect ("A" in Figure 9A) is defective, a positive effector should be removed at the same time. If the balance of "A" and "B" in Figure 9A is affected when negative regulators only are deleted, the accumulation of a poisonous factor might occur in this pathway. In the case of Nbp2, it is possible that A in Figure 9A is Ptcs, B in Figure 9A is Mkk1/2, and the poison is the hyperphosphorylation of the target of Ptcs. There could be another case in which, by inactivating one gene, the toxic gene products might accumulate and cause the growth arrest, in which case one should try to remove the toxic gene on deletion screening. We believe that this technique is a very useful genetic approach for the future.

Nbp2 signaling network: In this report, we isolated multicopy suppressors (*PTC1*, *PTC2*, *PTC4*, *MSB1*, *SKT5*) and deletion suppressors (*GRE1*, *SEF1*, *MKK1*, *MKK2*, *YFR016C*, *PYK2*) of the *NBP2* deficiency. Previous work has identified several genes that show a synthetic lethal/sick interaction with *nbp2*Δ (TONG *et al.* 2001) and a two-hybrid interaction with Nbp2 (ITO *et al.* 2000; UETZ *et al.* 2000). The signaling network of Nbp2 shown in Figure 9B contains four interactions (multicopy and deletion suppressor, synthetic lethal/sick, and two-hybrid interactions). *NBP2* has recently been shown to show a synthetic lethal/sick interaction with *bni1*Δ (TONG *et al.* 2001). We have noted that many of the genes interacting with *NBP2* also shared an interaction with *BNI1*. *BNI1*

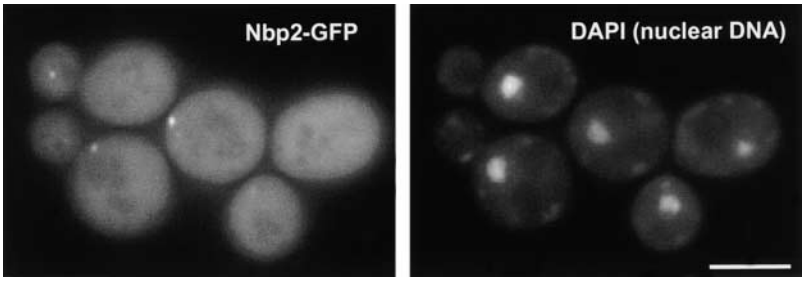


FIGURE 7.—Subcellular localization of Nbp2. The subcellular localization of a Nbp2-GFP fusion protein expressed from a multicopy plasmid (YEUpNBP2-GFP) was examined in a *nbp2*Δ strain (YKO214). Cells were grown in SD medium to an OD_{600} of 0.5–0.7 and were visualized using a fluorescence microscope. Bar, 5 μm.

A

Two-hybrid interactions

DNA Binding Domain	Transcription Activation domain	β-gal Activity
NAP1 (1-417)	-	0.081
	NBP1 (34-319)	3.35
	NBP2 (155-236)	10.03
SIR4	SIR4	22.30

B

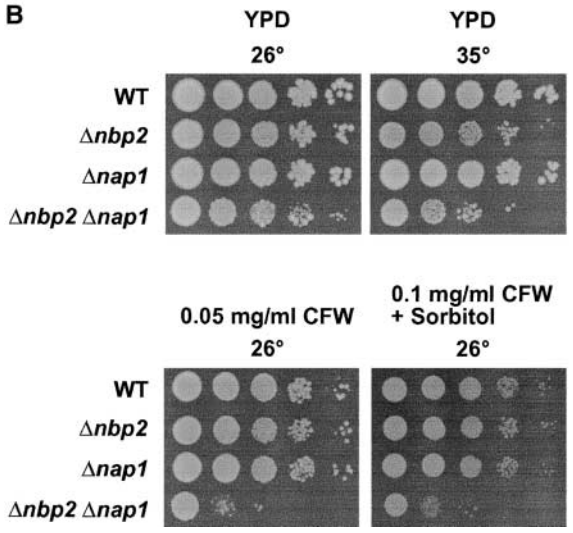
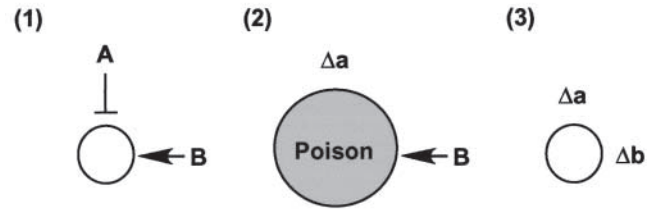


FIGURE 8.—Synthetic sick interaction between *NBP2* and *NAP1*. (A) Strain L40 carrying pBTM-NAP1 was transformed with pGAD, pGAD-NBP1, or pGAD-NBP2. Exponential-phase yeast cells were assayed for β-galactosidase activity as described by VOJTEC *et al.* (1993). Plasmids pBTM-SIR4 and pGAD-SIR4 were used as a positive control. (B) Yeast cells grown for 2 days on YPD medium were harvested, washed, normalized by OD_{600}, and spotted onto YPD, YPD supplemented with 0.05 mg/ml CFW, and YPD supplemented with 0.1 mg/ml CFW and 20% sorbitol in a series of five 10-fold dilutions. Plates were allowed to grow for 3 days at 26° or 35°. Isogenic yeast strains used were as follows: RAY (wild type), YKO213 (*nbp2*Δ), YKO227 (*nap1*Δ), and YKO240 (*nbp2*Δ *nap1*Δ). WT, wild type.

A



B

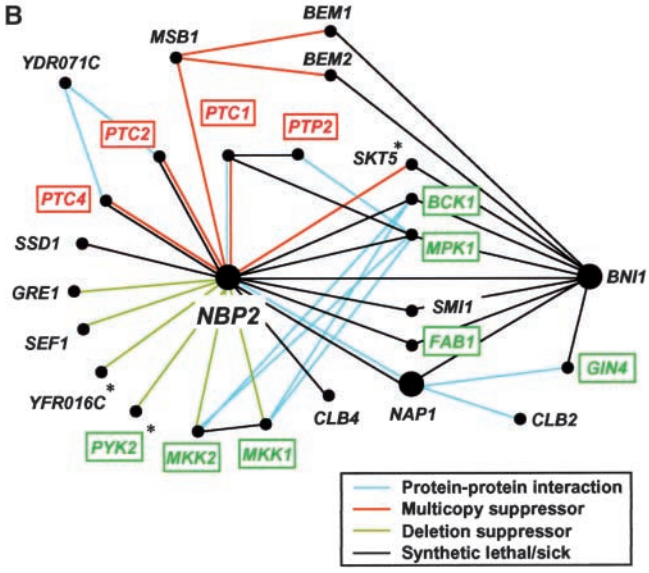


FIGURE 9.—The role of Nbp2. (A) Model for a deletion suppressor. "A" is a negative regulator. "B" is a positive regulator. (1) In wild-type cells, there is a balance between "A" and "B." (2) If "A" is deleted, the balance of "A" and "B" is lost. (3) When both "A" and "B" are deleted, there is a balance between "A" and "B" as in wild-type cells. (B) Genetic and biochemical interaction network of Nbp2. Genes are represented as nodes, and interactions are represented as edges that connect the nodes. All of the multicopy and deletion suppressor genes of the *NBP2* deficiency and part of the protein-protein and synthetic lethal/sick relationships are shown. Overproduction of *MSB1* partially suppresses *bem1* or *bem2* mutants. References for all the genes except our data can be found listed as synthetic lethal/sick interactions in TONG *et al.* (2001), in the Yeast Protein Database, and in the *Saccharomyces* Genome Database. Nbp2, directly or indirectly, interacts with protein phosphatase (red) and kinase (green). The interaction of Nbp2 and Ssd1 or Nap1 is synthetic sick. *SKT5*, *PYK2*, and *YFR016C* are isolated using only a *ssd1-d* background (asterisk).

encodes members of the highly conserved family that control the assembly of actin cables, which guide myosin motors to coordinate the polarized cell growth and spindle orientation (EVANGELISTA *et al.* 2002). As TONG *et al.* (2001) suggested, one of the Nbp2 functions might be cytoskeletal organization. We believe that the role of Nbp2 is as a general cellular signaling system (*e.g.*, cytoskeletal organization, cell wall maintenance, and cell cycle control). Deletion of *NBP2* does not apparently affect actin staining (K. OHKUNI and A. KIKUCH, unpublished data). We showed that *NBP2* is required for promoting cell wall integrity and is essential for mitotic growth at high temperatures. *NBP2*, which interacted and related with several genes (*MSB1*, *SMI1*, *FAB1*, *BEM1*, *BEM2*), is involved in cell polarity and cell wall organization. Furthermore, Nbp2 shows a synthetic sick interaction with Nap1, which binds to Clb2 (B-type cyclin; KELLOGG *et al.* 1995) and to Clb4 (B-type cyclin; TONG *et al.* 2001). Clearly, further studies are needed to more clearly determine the role of Nbp2 in the cellular signaling system.

Genetic screens in yeast have revealed that *NBP2* directly or indirectly interacts with protein phosphatases and kinases (Figure 9B). Previous studies demonstrate that Nap1 binds Gin4 kinase and works with Cla4 and

Elm1 kinase to control mitotic events (Sreenivasan and Kellogg 1999). Mammalian TAF1 proteins (α and β), a Nap1 family protein, have the inhibitory activity of type-2A protein phosphatase (Saito *et al.* 1999). It is interesting to note that the functions of Nap1 and Nbp2 are very similar, involving phosphorylation and dephosphorylation of proteins, which suggests that Nbp2 may be a part of Nap1's function through various signaling networks. We do not know yet what kind of proteins were phosphorylated or dephosphorylated as targets of the Nbp2 signaling network; however, a more precise understanding of the role of Nbp2 in these processes will emerge from further studies of the interactions of this protein.

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LITERATURE CITED

Altman, R., and D. Kellogg, 1997 Control of mitotic events by Nap1 and the Gin4 kinase. J. Cell Biol. **138:** 119–130.

Boles, E., F. Schulte, T. Miosga, K. Freidel, E. Schluter *et al.*, 1997 Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in Saccharomyces cerevisiae that is catalytically insensitive to fructose-1,6-bisphosphate. J. Bacteriol. **179:** 2987–2993.

Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer *et al.*, 1979 Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:** 17–24.

Burns, N., B. Grimwade, P. B. Ross-Macdonald, E. Y. Choi, K. Finberg *et al.*, 1994 Large-scale analysis of gene expression, protein localization, and gene disruption in Saccharomyces cerevisiae. Genes Dev. **8:** 1087–1105.

Cabib, E., and A. Duran, 1975 Simple and sensitive procedure for screening yeast mutants that lyse at nonpermissive temperatures. J. Bacteriol. **124:** 1604–1606.

Chang, L., S. S. Loranger, C. Mizzen, S. G. Ernst, C. D. Allis *et al.*, 1997 Histones in transit: cytosolic histone complexes and diacetylation of H4 during nucleosome assembly in human cells. Biochemistry **36:** 469–480.

Cheng, A., K. E. Ross, P. Kaldis and M. J. Solomon, 1999 Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. Genes Dev. **13:** 2946–2957.

Chien, C. T., P. L. Bartel, R. Sternglanz and S. Fields, 1991 The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:** 9578–9582.

Evangelista, M., D. Pruyne, D. C. Amberg, C. Boone and A. Bretscher, 2002 Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat. Cell Biol. **4:** 32–41.

Fonzi, W. A., and M. Y. Irwin, 1993 Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:** 717–728.

Herskowitz, I., 1995 MAP kinase pathways in yeast: for mating and more. Cell **80:** 187–197.

Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki *et al.*, 1993 MKK1 and MKK2, which encode Saccharomyces cerevisiae mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol. Cell. Biol. **13:** 3076–3083.

Ishimi, Y., H. Yasuda, J. Hirosumi, F. Hanaoka and M. Yamada, 1983 A protein which facilitates assembly of nucleosome-like structures in vitro in mammalian cells. J. Biochem. **94:** 735–744.

Ito, T., M. Bulger, M. J. Pazin, R. Kobayashi and J. T. Kadonaga, 1997 ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell **90:** 145–155.

Ito, T., K. Tashiro, S. Muta, R. Ozawa, T. Chiba *et al.*, 2000 Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc. Natl. Acad. Sci. USA **97:** 1143–1147.

Jiang, B., A. F. Ram, J. Sheraton, F. M. Klis and H. Bussey, 1995 Regulation of cell wall beta-glucan assembly: PTC1 negatively affects PBS2 action in a pathway that includes modulation of EXG1 transcription. Mol. Gen. Genet. **248:** 260–269.

Kaeberlein, M., and L. Guarente, 2002 *Saccharomyces cerevisiae MPT5* and *SSD1* function in parallel pathways to promote cell wall integrity. Genetics **160:** 83–95.

Kawase, H., M. Okuwaki, M. Miyaji, R. Ohba, H. Handa *et al.*, 1996 NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. Genes Cells **1:** 1045–1056.

Kellogg, D. R., and A. W. Murray, 1995 NAP1 acts with Clb2 to perform mitotic functions and to suppress polar bud growth in budding yeast. J. Cell Biol. **130:** 675–685.

Kellogg, D. R., A. Kikuchi, T. Fujii-Nakata, C. W. Turck and A. W. Murray, 1995 Members of the NAP/SET family of proteins interact specifically with B-type cyclins. J. Cell Biol. **130:** 661–673.

Levin, D. E., and B. Errede, 1995 The proliferation of MAP kinase signaling pathways in yeast. Curr. Opin. Cell Biol. **7:** 197–202.

Matsumoto, K., K. Nagata, M. Miyaji-Yamaguchi, A. Kikuchi and M. Tsujimoto, 1999 Sperm chromatin decondensation by template activating factor I through direct interaction with basic proteins. Mol. Cell. Biol. **19:** 6940–6952.

Mayer, B. J., 2001 SH3 domains: complexity in moderation. J. Cell Sci. **114:** 1253–1263.

Nagata, K., H. Kawase, H. Handa, K. Yano, M. Yamasaki *et al.*, 1995 Replication factor encoded by a putative oncogene, set, associated with myeloid leukemogenesis. Proc. Natl. Acad. Sci. USA **92:** 4279–4283.

Nagata, K., S. Saito, M. Okuwaki, H. Kawase, A. Furuya *et al.*, 1998 Cellular localization and expression of template-activating factor I in different cell types. Exp. Cell Res. **240:** 274–281.

Ohkuni, K., and I. Yamashita, 2000 A transcriptional autoregulatory loop for KIN28-CCL1 and SRB10-SRB11, each encoding RNA polymerase II CTD kinase-cyclin pair, stimulates the meiotic development of S. cerevisiae. Yeast **16:** 829–846.

Ohkuni, K., K. Shirahige and A. Kikuchi, 2003 Genome-wide expression analysis of NAP1 in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. **306:** 5–9.

Paravicini, G., M. Cooper, L. Friedli, D. J. Smith, J. L. Carpentier *et al.*, 1992 The osmotic integrity of the yeast cell requires a functional PKC1 gene product. Mol. Cell. Biol. **12:** 4896–4905.

Parent, S. A., C. M. Fenimore and K. A. Bostian, 1985 Vector systems for the expression, analysis and cloning of DNA sequences in S. cerevisiae. Yeast **1:** 83–138.

Poch, O., 1997 Conservation of a putative inhibitory domain in the GAL4 family members. Gene **184:** 229–235.

Saito, S., M. Miyaji-Yamaguchi, T. Shimoyama and K. Nagata, 1999 Functional domains of template-activating factor-I as a protein phosphatase 2A inhibitor. Biochem. Biophys. Res. Commun. **259:** 471–475.

Shimizu, Y., T. Akashi, A. Okuda, A. Kikuchi and K. Fukui, 2000 NBP1 (Nap1 binding protein 1), an essential gene for G2/M transition of Saccharomyces cerevisiae, encodes a protein of distinct sub-nuclear localization. Gene **246:** 395–404.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Sreenivasan, A., and D. Kellogg, 1999 The elm1 kinase functions in a mitotic signaling network in budding yeast. Mol. Cell. Biol. **19:** 7983–7994.

Sutton, A., D. Immanuel and K. T. Arndt, 1991 The SIT4 protein phosphatase functions in late G1 for progression into S phase. Mol. Cell. Biol. **11:** 2133–2148.

Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader *et al.*, 2001 Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science **294:** 2364–2368.

Uesono, Y., A. Fujita, A. Toh-e and Y. Kikuchi, 1994 The MCS1/SSD1/SRK1/SSL1 gene is involved in stable maintenance of the chromosome in yeast. Gene **143:** 135–138.

Uesono, Y., A. Toh-e and Y. Kikuchi, 1997 Ssd1p of Saccharomyces cerevisiae associates with RNA. J. Biol. Chem. **272:** 16103–16109.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature **403:** 623–627.

Vojtek, A. B., S. M. Hollenberg and J. A. Cooper, 1993 Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:** 205–214.

Walter, P. P., T. A. Owen-Hughes, J. Cote and J. L. Workman, 1995 Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. Mol. Cell. Biol. **15:** 6178–6187.

Warmka, J., J. Hanneman, J. Lee, D. Amin and I. Ota, 2001 Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. Mol. Cell. Biol. **21:** 51–60.

Yang, P., R. Pimental, H. Lai and S. Marcus, 1999 Direct activation of the fission yeast PAK Shk1 by the novel SH3 domain protein, Skb5. J. Biol. Chem. **274:** 36052–36057.

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