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CAC3 (*MSI1*) Suppression of *RAS2*^{G19V} Is Independent of Chromatin Assembly Factor I and Mediated by *NPR1*

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Cac3p/Msi1p, the *Saccharomyces cerevisiae* homolog of retinoblastoma-associated protein 48 (RbAp48), is a component of chromatin assembly factor I (CAF-I), a complex that assembles histones H3 and H4 onto replicated DNA. *CAC3* overexpression also suppresses the *RAS*/cyclic AMP (cAMP) signal transduction pathway by an unknown mechanism. We investigated this mechanism and found that *CAC3* suppression of *RAS*/cAMP signal transduction was independent of either *CAC1* or *CAC2*, subunits required for CAF-I function. *CAC3* suppression was also independent of other chromatin-modifying activities, indicating that Cac3p has at least two distinct, separable functions, one in chromatin assembly and one in regulating *RAS* function. Unlike Cac1p, which localizes primarily to the nucleus, Cac3p localizes to both the nucleus and the cytoplasm. In addition, Cac3p associates with Npr1p, a cytoplasmic kinase that stablizes several nutrient transporters by antagonizing a ubiquitin-mediated protein degradation pathway. Deletion of *NPR1*, like overexpression of Cac3p, suppressed the *RAS*/cAMP pathway. Furthermore, *NPR1* overexpression interfered with the ability of *CAC3* to suppress the *RAS*/cAMP pathway, indicating that extra Cac3p suppresses the *RAS*/cAMP pathway by sequestering Npr1p. Deletion of *NPR1* did not affect the quantity, phosphorylation state, or localization of Ras2p. Consistent with the idea that Npr1p exerts its effect on the *RAS*/cAMP pathway by antagonizing a ubiquitin-mediated process, excess ubiquitin suppressed both the heat shock sensitivity and the sporulation defects caused by constitutive activation of the *RAS*/cAMP pathway. Thus, *CAC3*/*MSI1* regulates the *RAS*/cAMP pathway via a chromatin-independent mechanism that involves the sequestration of Npr1p and may be due to the increased ubiquitination of an Npr1p substrate.

Chromatin assembly factor I (CAF-I) is a complex of three proteins that has been purified from both mammalian and yeast cells that assembles histones H3 and H4 onto newly replicated DNA (24, 38). The chromatin assembly complex (CAC) is the combined complex of CAF-I with histones H3 and H4 (48). The three CAF-I proteins from *Saccharomyces cerevisiae* are designated Cac1p, Cac2p, and Cac3p and correspond to the human CAF-I proteins p150, p60, and p48, respectively. Deletion of any one of the three *CAC* genes in *S. cerevisiae* results in multiple phenotypes, including the derepression of telomere-adjacent genes, mislocalization of the telomere-binding protein Rap1p, and an increase in sensitivity to UV radiation (11, 24). However, deletion of any one or all three of the *CAC* genes is not lethal, indicating that there must be other activities in *S. cerevisiae* capable of chromatin assembly. Loss of one of the *CAC* genes coupled with loss of one of the *HIR* genes (which control histone H2A and H2B function) leads to synergistic defects in chromatin structure and decreased growth rates (22, 33).

CAC1 and *CAC2* mRNAs are coordinately regulated, with expression peaking in the G_1 phase of the cell cycle (39). In contrast, *CAC3* mRNA levels do not change through the cell cycle (39). All three Cac proteins copurify (23, 24, 28), but the majority of p48 in human cells is found in a large complex that does not include p150 or p60 (28). Vertebrate p48 also copurifies with the histone deacteylase HDAC1 (43) and with pRb, the product of the retinoblastoma susceptibility gene (32), which acts as a tumor suppressor. The closest Cac3p homolog in *S. cerevisiae* is Hat2p, the subunit of histone acetyltransferase I that is necessary for association with histones H3 and H4 (31). Viewed together, these data suggest that Cac3p/p48 plays multiple roles in the deposition and modification of histones (34).

Cac3p appears to have at least one role unrelated to its function in chromatin assembly and/or histone modification. *CAC3* was originally isolated as *MSI1*, a multicopy suppressor of *IRA1* (35), which is a negative regulator of the *RAS*/cyclic AMP (cAMP) pathway. High-copy *CAC3* reduces cAMP levels in *ira1* and *RAS2*^{G19V} strains, mitigating the heat shock sensitivity and sporulation deficiency of these strains. High-copy *CAC3* also suppresses *snf1* and *snf4* mutations by decreasing cAMP levels (18). Overexpression of human p48, like *CAC3*/*MSI1*, can suppress the *RAS*/cAMP pathway in *S. cerevisiae* (32, 35).

The *RAS*/cAMP pathway has been well characterized in the

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yeast *Saccharomyces*. Two genes in *S. cerevisiae*, *RAS1* and *RAS2*, are structural and functional homologs of human *ras* genes (8, 20), oncogenic mutations of which are found in 90% of pancreatic, 50% of colon, and 30% of lung adenocarcinomas as well as in 50% of thyroid tumors and 30% of myeloid leukemias (3). In yeast cells, Ras controls the metabolic state and alters the stress response of the cell through modulation of cAMP levels (5). Cdc25p stimulates Ras activity by promoting the exchange of GDP for GTP through the stabilization of Ras in a nucleotide-free state (16). Ira1p and Ira2p negatively regulate Ras proteins by stimulating their intrinsic GTPase activity (29). The $RAS2^{G19V}$ allele, which is analogous to the most common oncogenic mutation in human cancers (3), encodes a protein that binds GTP normally but fails to hydrolyze it (30), resulting in constitutively active Ras protein. Activated Ras protein stimulates adenylyl cyclase (Cyr1p/Cdc35p), thereby promoting production of cAMP. cAMP binds to Bcy1p, the negative regulatory subunit of protein kinase A (PKA), and disassociates it from the catalytic subunit (encoded by any one of the three genes *TPK1*, *-2*, or *-3*) to yield enhanced kinase activity. PKA activation leads to glycogen utilization, increased glycolysis, the induction of many growth-related genes (5), and reduced expression of genes encoding heat shock proteins Hsp72, Hsp41 (37), and Hsp12 (47). Cells containing an activated *RAS*/cAMP pathway (those containing $RAS2^{G19V}$, *bcy1*Δ, or *ira1*Δ mutations) mate poorly, contain low levels of storage carbohydrates, and are sensitive to transient heat shock.

The mechanism by which Cac3p suppresses the heat shock sensitivity and the sporulation defect of cells with an activated *RAS*/cAMP pathway is not known. High-copy *CAC3* suppresses phenotypes caused by the constitutively active $RAS2^{G19V}$ allele or by deletion of the negative regulator *IRA1* but does not suppress these phenotypes when the pathway is activated by deletion of *BCY1* (35). We investigated the mechanism by which Cac3p overproduction suppresses the $Ras2p^{G19V}$ oncoprotein. We found that the ability of *CAC3* overexpression to suppress the *RAS*/cAMP pathway is independent of its role in CAF-I-mediated chromatin assembly and its putative role in histone modification activities. This result is similar to the recently published results of Zhu et al. (51). Consistent with this, a significant proportion of Cac3p resides in the cytoplasm, in contrast to Cac1p, which is primarily nuclear. We identified Npr1p as a protein that physically interacts with Cac3p and found that loss of Npr1p function suppresses the *RAS*/cAMP pathway in a manner indistinguishable from *CAC3* overexpression. The Npr1p kinase is known to antagonize the ubiquitin-mediated inactivation of several transporter proteins; similarly, we have found that overexpression of polyubiquitin is also capable of suppressing $RAS2^{G19V}$-induced heat shock sensitivity. Our results suggest that *CAC3* sequesters and thereby inactivates the Npr1p kinase, which effectively reduces the activity of the *RAS*/cAMP pathway.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* strains XL1-Blue and DH5α were used for all standard plasmid preparations and manipulations (1). pML9 for the disruption of *NPR1* was provided by J. Heitman (26). pJW192, encoding a Ras2p-green fluorescent protein (GFP) fusion, was provided by J. Rine (4). p*CUP1*-myc-*UBI4* was provided by M. Hochstrasser (9). p*bcy1*::*URA3* has been described (45). Triple-hemagglutinin (HA) epitope-tagged Npr1p carrying a mutation that presumably blocks its kinase activity was a generous gift from Yu Jiang. This mutation, D579E, was created on the basis of homology to mutations known to inactivate other kinases, although the effect of the mutation on Npr1 kinase activity has not been demonstrated.

pGBT9-*CAC3* was amplified from yeast strain Y294 by PCR amplification using the oligonucleotides 5′-GGCCGGGGATCCATGAATCAGTGCGCGAAGG-3′ and 5′-GGGCCCGTCGACTCACGAATGTCCAACAAGGTTTCC-3′. The PCR product was cloned into pGBT9C which had been digested with *Bam*HI and *Sal*I. pGBT9C is the pGBT9 vector which has been altered in the reading frame of the multiple cloning site by the addition of two additional guanine residues before the *Eco*RI site and was a generous gift from Clint McDonald. YEp55-*CAC3* was constructed by amplifying the *CAC3* gene from pGBT9-*CAC3* by PCR using the oligonucleotides 5′-GGCCGGGTCGACATGAATCAGTGCGCGAAGG-3′ and 5′-GGGCCCGGATCCTCACGAATGTCCAACAAGGTTTCC-3′ and was cloned into YEp55S that had also been digested with *Sal*I and *Bam*HI. The multiple cloning site of this vector had been modified through the introduction of a *Sal*I restriction site by Corey Davis and was a gift from him. pRS406-Ras2Val19 was constructed by isolating the 2.1-kb genomic *Eco*RI-*Hin*dIII fragment containing the $RAS2^{G19V}$ allele and cloned into pRS406 that had been digested with *Eco*RI and *Hin*dIII. pGAD1-*CAC1* was cloned from a yeast genomic library constructed by Stan Fields and was a generous gift from Mark Rose.

NPR1 (with its start and stop codons) was amplified from W303 genomic DNA by PCR and cloned into pCR-II (Invitrogen). The *Xho*I-*Hin*dIII fragment containing the *NPR1* gene was subcloned into pRSET-B (Invitrogen) to yield pRSET-*NPR1*. pRSET-*NPR1* was digested with *Xba*I and the fragment was cotransformed into *S. cerevisiae* with pGalSET984 (10) linearized with *Xho*I. In vivo recombination between these two DNA fragments yielded pGalSET-*NPR1* (10). Similarly, *CAC3* was amplified from W303 genomic DNA by PCR and cloned into pCR-II. The *Bam*HI-*Hin*dIII fragment of this plasmid containing the *CAC3* gene was subcloned into pRSET-B. The *Xba*I fragment containing *CAC3* and *Xho*I-linearized pGalSET985 were cotransformed into yeast cells, selecting for in vivo recombination. Immunoblotting confirmed that galactose induction of strains carrying either pGalSET-*NPR1* or pGalSET-*CAC3* generated an epitope-tagged protein of the expected size (data not shown). The protein encoded by YEp55-*CAC3* was tagged at the carboxy terminus with GFP, using the PCR-mediated technique described by Longtine et al. (25). *URA3* was integrated adjacent to the left telomere of chromosome VII as described (12).

The yeast strains used in this study are listed in isogenic groups in Table 1. Strains were grown in standard laboratory SD complete (SDC) medium with the appropriate amino acid dropouts (15). Genetic crosses, sporulation, dissection, and transformation were performed as described (15). Auxotrophic markers were swapped as described (7). The [rho$^-$] strain was made by growth of the parental strain in the presence of ethidium bromide (25 μg/ml).

Heat shock and sporulation assays. Yeast cells were grown to saturation on appropriate dropout medium with either 2% glucose or 2% galactose present as the carbon source. Cells were collected by centrifugation, washed once, and resuspended in water. Aliquots were incubated at 55°C for the time periods indicated. After cooling to room temperature, cells were serially diluted 10-fold, plated, and incubated at 30°C for 2 days. Resulting colonies were counted and normalized to the number of viable cells in an aliquot that was not exposed to 55°C. To determine sporulation efficiency, diploid strains were grown overnight in appropriate dropout medium supplemented with 2% galactose to induce the expression of genes under control of the *GAL1* promoter, as appropriate. Cells were collected by centrifugation, washed twice, and allowed to sporulate for 3 days in 1% potassium acetate at room temperature, with shaking. Diploids and tetrads (at least 300 cells of each strain) were counted by microscopic observation.

Two-hybrid screen. An *S. cerevisiae* genomic DNA two-hybrid library in pGAD1, -2, or -3 (6) was kindly provided by P. Siliciano (University of Minnesota) and transformed into HF7c containing pGBT9-*CAC3* as bait. Transformed cells which contained interacting fusion proteins were selected by plating on SDC lacking Leu, Trp, and His and supplemented with 5 mM 3-amino-1,2,4-triazole (3AT). Transformants that contained *GAL4* were identified by PCR and discarded. Surviving yeast strains were cured of either plasmid to ensure that growth on the selective medium was dependent on the presence of both plasmids. Inserts in pGAD were amplified directly from the yeast strain by PCR using primers flanking the multiple cloning site. Amplified DNA was sequenced using a nested primer.

Coimmunoprecipitation. Yeast strains YSJ401, YSJ402, and YSJ403 were grown in SC lacking Leu and Trp containing 2% galactose for 2 days. Cells were collected by centrifugation and washed once with water and once with 10mM sodium azide. Cell pellets were then frozen at −70°C. Cells were then resuspended in 200 μl of buffer C (20 mM HEPES-KOH [pH 7.4], 150 mM NaCl, 1

TABLE 1. Yeast strains used

Isogenic group and strain	Relevant characteristic(s)	Source or reference
W303		
YJB195	*MAT***a** *ura3-1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1*	Berman lab
YJB209	*MAT*α *ura3-1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1*	Berman lab
YJB334	*MAT***a**/α *ura3-1/ura3-1 ade2-1/ ade2-1 his3-11/ his3-11 leu2-3,112/leu2-3,112 can1-100/ can1-100 trp1-1/trp1-1*	Berman lab
YJB1358	YJB209 *cac1-1*	11
YJB1599	YJB209 *cac2*::*TRP1*	24
YJB1786	YJB209 *cac3*::*hisG VIIL*::*URA3-TEL*	This study
YJB2235	YJB195 *URA3*::*RAS2*^{G19V}	This study
YJB2237	YJB209 *cac1-1 URA3*::*RAS2*^{G19V}	This study
YJB2320	YJB195 *URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2322	YJB209 *cac1-1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2543	YJB195 *sir3*::*TRP1*	Berman lab
YJB2546	YJB195 *gcn5*::*URA3*	Berman lab
YJB3135	YJB195 *gcn5*::*ura3*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB3141	YJB195 *gcn5*::*ura3*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB3554	YJB195 *npr1*::*LEU2 bcy1*::*URA3*	This study
YJB3563	YJB209 *cac2*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB3610	YJB209 *VIIL*::*URA3-TEL* pGalSET	This study
YJB3670	YJB195 *sch9*::*TRP1* YCP50-*RAS2*$^{G18A\ G19V}$	50a
YJB3674	YJB195 *sch9*::*TRP1* YCP50-*RAS2*$^{G18A\ G19V}$ YEP55-*CAC3*	This study
YJB3712	YJB195 *npr1*::*LEU2*	This study
YJB3721	YJB334 *ura3-1*/*URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB3723	YJB195 *npr1*::*LEU2 URA3*::*RAS2*^{G19V}	This study
YJB3724	YJB2235 *pCUP1-myc-UB14*	This study
YJB3737	YJB334 *ura3-1*/*URA3*::*RAS2*^{G19V}	This study
YJB3739	YJB334 YEp55-*CAC3*	This study
YJB3763	YJB195 *sir3*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB3773	YJB195 *sir3*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB3785	*MAT***a** *his3 Leu2-3, 112 trp1 ura3-1*	4
YJB3791	YJB334 *ura3-1*/*URA3*::*RAS2*^{G19V} *npr1*::*LEU2*/*npr1*::*LEU2*	This study
YJB3792	YJB334 *npr1*::*LEU2*/*npr1*::*LEU2*	This study
YJB3863	YJB195 *URA3*::*RAS2*^{G19V} pGalSET-*NPR1*	This study
YJB3875	YJB195 *URA3*::*RAS2*^{G19V} pGalSET-*NPR1* pGalSET-CAC3	This study
YJB3876	YJB209 *cac2*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB3896	YJB3785 pGFP-*RAS2*	This study
YJB3897	YJB3785 *npr1*::*LEU2* pGEP-*RAS2*	This study
YJB3898	YJB3785 YEp55-*CAC3* pGFP-*RAS2*	This study
YJB3954	YJB195 pGalSET-*NPR1*	This study
YJB4034	YJB3785 YEp55-*CAC3*	This study
YJB4317	YJB4034 YEp55-*CAC3-GEP*	This study
YJB4506	YJB4317 YEp55-*CAC3-GFP* [rho$^-$]	This study
YJB4632	YJB209 *gpa2*::*TRP1*	50a
YJB4635	YJB209 *gpr1*::*HIS3*	50a
YJB5235	YJB195 *URA3*::*RAS2*^{G19V} *gpa2*::*TRP1* YEp55-*CAC3*	This study
YJB5243	YJB195 *URA3*::*RAS2*^{G19V} *gpa2*::*TRP1*	50a
YJB5237	YJB195 *URA3*::*RAS2*^{G19V} *gpr1*::*HIS3* YEp55-*CAC3*	This study
YJB5245	YJB195 *URA3*::*RAS2*^{G19V} *gpr1*::*HIS3*	50a
YJB5522	YJB3723 pCUP1-myc-UB14	This study
YJB5523	YJB3737 pCUP1-myc-UB14	This study
YSJ328	YJB3954 *VIIL*::*URA3-TEL*	This study
Y2799	*MAT*α *ura3-1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 npr1*::*LEU2* pRS424 pGalSET-*CAC3*	This study
Y2800	*MAT*α *ura3-1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 npr1*::*LEU2* pRS424-3HA-*NPR1* pGalSET-*CAC3*	This study
Y2801	*MAT*α *ura3-1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 npr1*::*LEU2* pRS424-3HA-*NPR1*-KD pGalSET-*CAC3*	This study
YPH49/50		
YJB1583	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-ars1 his3-200 leu2*Δ*1*	31
YJB1584	YJB1583 *hat1*::*HIS3*	31
YJB1585	YJB1583 *hat2*::*TRP1*	31
YJB1586	YJB1583 *hat1*::*HIS3 hat2*::*TRP1*	31
YJB2349	YJB1583 *URA3*::*RAS2*^{G19V}	This study
YJB2350	YJB1583 *hat1*::*HIS3 URA3*::*RAS2*^{G19V}	This study
YJB2366	YJB1583 *URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2367	YJB1583 *hat1*::*HIS3 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2648	YJB1583 *hat2*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB2658	YJB1583 *hat2*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2672	YJB1583 *hat1*::*HIS3 hat2*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB2673	YJB1583 *hat1*::*HIS3 hat2*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2697	YJB1583 *bcy1*::*URA3*	This study
YJB2781	YJB1583 *bcy1*::*URA3* YEp55-*CAC3*	This study
YDS3		
YJB2431	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	35a
YJB2432	YJB2431 *hda1*::*TRP1*	35a
YJB2433	YJB2431 *rpd3*::*LEU2*	35a
YJB2434	YJB2431 *hda1*::*TRP1 rpd3*::*LEU2*	35a
YJB2474	YJB2431 *URA3*::*RAS2*^{G19V}	This study
YJB2475	YJB2431 *hda1*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB2476	YJB2431 *rpd3*::*leu2*::*HIS3 URA3*::*RAS2*^{G19V}	This study
YJB2474	YJB2431 *hda1*::*TRP1 rpd3*::*leu2*::*HIS3 URA3*::*RAS2*^{G19V}	This study
YJB2529	YJB2431 *URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2530	YJB2431 *rpd3*::*leu2*::*HIS3 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2531	YJB2431 *hda1*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB2532	YJB2431 *hda1*::*TRP1 rpd3*::*leu2*::*HIS3 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
HF7c		
YJB1325	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal180-538 LYS2*::*GAL1-HIS3 URA3*::*(GAL4* 17-mers$)_3$-*CYC1-lacZ*	Clontech Inc.
YJB3197	YJB1325 pGBT9-*Cac3p* pGAD1-*CAC1*	This study
YJB3438	YJB1325 pGBT9-*Cac3p* pGAD1-*NPR1*$_{561–605}$	This study
L1356		
YJB2136	*MAT***a** *ura3-52 his3-200 leu2-1 met8-1 ilv1-1 trp1-901 cac3-878 lys2*::*his3*Δ*4*	33
YJB2137	YJB2136 *hir3*::*LEU2*	33
YJB2998	YJB2136 *hir3*::*leu2*::*TRP1 URA3*::*RAS2*^{G19V}	This study
YJB2999	YJB2136 *hir3*::*leu2*::*TRP1 URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
YJB3016	YJB2136 *URA3*::*RAS2*^{G19V}	This study
YJB3017	YJB2136 *URA3*::*RAS2*^{G19V} YEp55-*CAC3*	This study
Σ1278b		
YJB2444	*MAT***a**/α *ura3-52/ura3-52*	24a
YJB3514	YJB2444 pGalSET	This study
YJB3515	YJB2444 pGalSET-*CAC3*	This study
YJB5723	YJB5724 *npr1*::*LEU2*/*npr1*::*LEU2*	26
YJB5724	*MAT***a**/α *ura3-52/ura3-52*	26

mM EDTA, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride plus 1 μg of pepstatin A, 0.5 μg of leupeptin, and 2 μg of aprotinin per ml) and lysed by vortexing with glass beads. Buffer C (200 μl) was then added, and unlysed cells, cellular debris, and glass beads were removed by centrifugation. Lysate (70 μg in 450 μl) was added to 20 μl of anti-HA affinity matrix (Roche) that had been blocked with bovine serum albumin (BSA, 2 mg/ml) in buffer C. Loading buffer (5 × sodium dodecyl sulfate [SDS]) was added to an aliquot of the input as a control. The lysate was allowed to bind to the affinity matrix for 3 h at 4°C. The affinity beads and bound proteins were pelleted by centrifugation, and the supernatant was retained. The 5 × SDS loading buffer was added to an aliquot of

the supernatant as a control. Beads were then washed four times with buffer C. After the final wash, beads were boiled for 3 min in 50 μl of 1 × SDS sample buffer, and an aliquot (15 μl) of each sample was loaded onto an 8% polyacrylamide–SDS gel and subjected to polyacrylamide gel electrophoresis (PAGE). Samples were then transferred onto a polyvinylidene difluoride membrane and probed with monoclonal antibodies against the T7 epitope (Novagen) or the HA epitope (12CA5). Immunoreactivity was detected using ECL (Amersham).

Microscopy and pseudohyphal assays. To determine the subcellular localization of Cac3p-GFP, YJB4506 was grown in SDC lacking Trp and supplemented with 2% raffinose, 0.5% galactose, and 10 ng of DAPI (4′,6′-diamidino-2-phenylindole) for 4 h at 30°C. Cells were viewed using a Nikon Eclipse E800 photomicroscope equipped with differential interference contrast and fluorescence optics using a 100 × 1.3-numerical-aperture plan apo objective. Digital images were collected using a CoolCam liquid-cooled, three-chip color charge-coupled device camera (Cool Camera Company, Decatur, Ga.) and captured to a Pentium II 300-MHz personal computer using Image Pro Plus version 4.0 software (Media Cybernetics, Silver Spring, Md.). Pseudohyphal growth was assayed as described by Lorenz and Heitman (26). Σ1278b strains carrying either a *CAC3* overexpression plasmid or the parental plasmid were grown overnight in either glucose or galactose. Cells were then plated on limiting nitrogen medium with either glucose (SLAD) or raffinose and galactose (SLADG) as a carbon source (26) and grown for 3 days at 30°C before being photographed.

Metabolic labeling and immunoprecipitation. ^{32}P metabolic labeling and Ras2p immunoprecipitation were performed essentially as described by Whistler and Rine (50). In brief, wild-type or *npr1*Δ cells were grown overnight in YPAD medium, washed in SDC low-phosphate medium, and grown in 10 ml of this medium at 30°C for 2 h. Then 2 mCi of $H_3{}^{32}PO_4$ (ICN) was added, and the culture was incubated at 30°C for an additional 3 h. Cells were collected by centrifugation, washed in NLB buffer (50 mM Tris [pH 7.5], 20 mM $MgCl_2$, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride) and stored at −70°C overnight. Cells were resuspended in 0.5 ml of NLB and lysed by bead beating. Unlysed cells and cell debris were removed by centrifugation. BSA-treated charcoal was added to the lysates, which were vortexed and centrifuged several times to remove all traces of the charcoal. Then 20 μl of either protein A-agarose or anti-Ras2-agarose (clone Y13-259; Calbiochem) was added to the lysate and mixed at 4°C for 2 h. Beads were washed three times with NLB and three times with NLB without detergent. Proteins were eluted from the beads by incubating at 70°C for 10 min in 20 μl of SDS loading buffer. Proteins were separated by SDS-PAGE through a 9% gel, which was fixed and dried, and and bands were quantitated by PhosphoImager analysis (Molecular Dynamics).

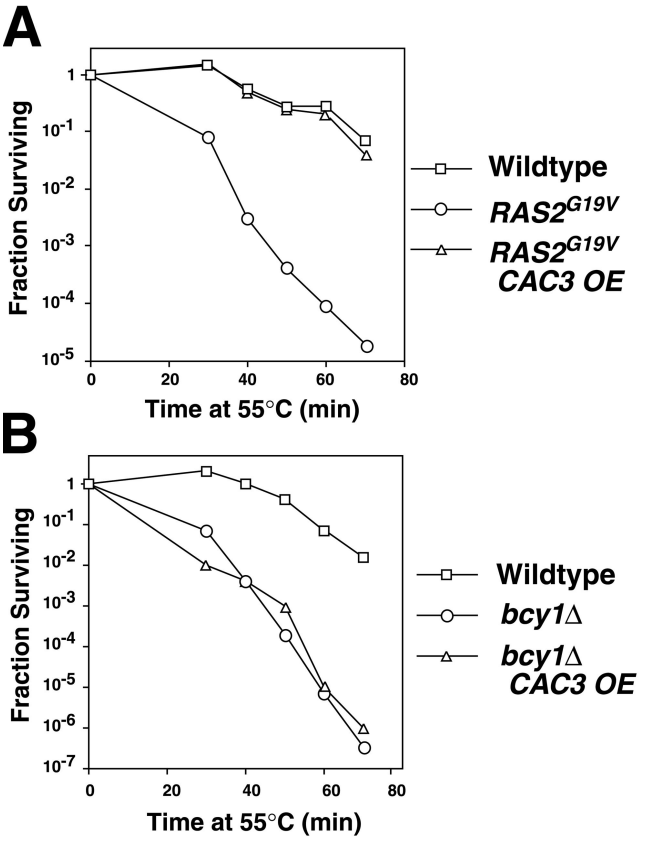

FIG. 1. *CAC3* overexpression suppresses the *RAS*/cAMP signal transduction pathway between RAS and PKA. The heat shock resistance of (A) isogenic wild-type (YJB195), *RAS2*^{G19V} (YJB2235), and *RAS2*^{G19V} *CAC3*-over expressing (OE) (YJB2320) strains and (B) isogenic wild-type (YJB1583), *bcy1*Δ (YJB2697), and *bcy1*Δ *CAC3*-overexpressing (YJB2781) strains was determined by incubation at 55°C for the indicated time periods as described in Materials and Methods.

RESULTS

***CAC3* suppression of the *RAS*/cAMP pathway is independent of CAF-I.** To understand the molecular mechanism by which *CAC3* overexpression affects the *RAS*/cAMP pathway, we first established a quantitative assay for heat shock sensitivity, a phenotype caused by activation of the *RAS*/cAMP pathway. Cells were grown to saturation, aliquoted and incubated at 55°C for different lengths of time, cooled, serially diluted, and plated on nonselective medium. Resulting colonies were counted after 2 days at 30°C. Yeast cells carrying the dominant *RAS2*^{G19V} allele have a constitutively activated *RAS*/cAMP pathway (30) and are sensitive to heat shock (Fig. 1A). Overexpression of *CAC3* suppressed the heat shock sensitivity of a strain carrying the *RAS2*^{G19V} allele (35) (Fig. 1A), restoring wild-type heat shock resistance. In contrast, heat shock sensitivity caused by deletion of *BCY1*, which encodes the negative regulator of PKA, was not suppressed by *CAC3* overexpression (35) (Fig. 1B). Thus, our quantitative heat shock sensitivity assay confirmed the reported ability of *CAC3* overexpression to suppress the *RAS*/cAMP pathway when it is activated by a *RAS2*^{G19V} allele but not when it is activated by *BCY1* deletion.

To determine if the CAF-I complex mediates *CAC3* suppression of the *RAS*/cAMP pathway, perhaps by affecting the expression of one or more genes that alter *RAS*/cAMP signal transduction, we asked if *CAC3* overexpression could suppress the *RAS*/cAMP pathway in the absence of functional CAF-I. We found that loss of *CAC1* function did not affect the heat shock sensitivity of *RAS2*^{G19V} cells and *CAC3* overexpression suppressed the heat shock sensitivity of *cac1-1 RAS2*^{G19V} cells (Fig. 2A). Similarly, deletion of *CAC2* had no effect on the heat shock sensitivity of *RAS2*^{G19V} cells or on the ability of *CAC3* overexpression to suppress the heat shock sensitivity (Fig. 2B). Thus, the ability of *CAC3* to affect the *RAS*/cAMP pathway did not require the presence of active CAF-I and must be independent of CAF-I-mediated chromatin assembly function.

The ability of Cac3p to function independently of Cac1p and Cac2p is supported by the previous observations that the cell cycle regulation of *CAC3* transcripts is different from that of *CAC1* and *CAC2* transcripts (39) and that Cac3p/p48 does not always copurify with Cac1p and Cac2p (28). Consistent with this, we found that Cac3p-GFP had a localization pattern different from that of Cac1p: Cac3p-GFP appeared to be diffuse and localized throughout the nucleus and cytoplasm (but not in the vacuoles) (Fig. 2C) during all stages of the cell cycle (S. Enomoto and J. Berman, unpublished data). In contrast, epitope-tagged Cac1p localized primarily to large foci within

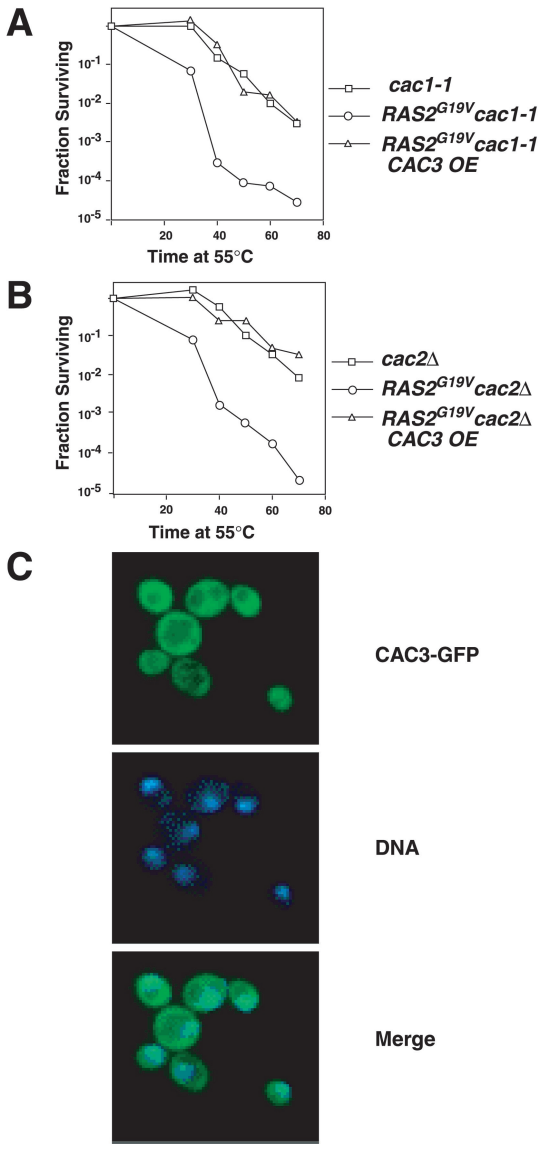

FIG. 2. *CAC1* and *CAC2* are not required for *CAC3* to suppress the *RAS*/cAMP pathway. The heat shock sensitivity of (A) *cac1-1* (YJB1358), *RAS2*^{G19V} *cac1-1* (YJB2237), *RAS2*^{G19V} *cac1-1 CAC3*-overexpressing (OE) (YJB2322) or (B) *cac2*Δ (YJB1599), *RAS2*^{G19V} *cac2*Δ (YJB3563), and *RAS2*^{G19V} *cac2*Δ *CAC3*-overexpressing (YJB3876) was determined as described for Fig. 1. (C) Cells carrying Cac3p-GFP (YJB4034) were stained with DAPI and analyzed by fluorescence microscopy to observe both GFP (top panel) and DNA (middle panel). The merged image (bottom panel) shows that Cac3p-GFP localizes throughout the cytoplasm and nucleus. Darker regions correspond to the vacuoles.

the nucleus, even when expressed from a high-copy-number vector (11).

***CAC3* suppression of the *RAS*/cAMP pathway is independent of several histone-modifying activities.** Cac3p and other p48-related proteins are involved in several chromatin-related functions. For example, *CAC3* has been shown to antagonize the Sin3p-Rpd3p histone deacetylation complex (42). Additionally, Cac3p has high sequence similarity to RbAp48p and RbAp46, which are subunits of histone modification enzymes such as histone deacetylase I (HDAC1) and histone acetylases (43, 49). Therefore, we asked if Cac3p affects the *RAS*/cAMP pathway through its association with histone deacetylases such as Rpd3p and Hda1p. The heat shock sensitivity of *RAS2*^{G19V} strains lacking *RPD3* and/or *HDA1* did not differ from that of otherwise wild-type *RAS2*^{G19V} strains (Table 2). Furthermore, there was no difference in the degree to which *CAC3* overexpression suppressed the heat shock sensitivity of these strains (Table 2).

Based on the similarity between Cac3p and Hat2p, a factor that facilitates the association of Hat1p with histones H3 and H4 (31), we investigated the ability of *CAC3* to suppress *RAS2*^{G19V}-induced heat shock sensitivity in strains lacking genes encoding histone acetyltransferase components. The heat shock sensitivity of *RAS2*^{G19V} strains carrying deletions in *HAT1*, *HAT2*, *HAT1* and *HAT2*, or *GCN5* was measured in the presence and absence of a plasmid overexpressing *CAC3*. In all of these strains, heat shock sensitivity and *CAC3* suppression of this sensitivity were not significantly different from what was found in the isogenic wild-type *RAS2*^{G19V} strain (Table 2). In addition, we asked if deletion of *HIR3*, which encodes a histone transcriptional regulator that has synergistic effects with *CAC* mutants, or of *SIR3*, which encodes a component of silent chromatin, would affect *RAS2*^{G19V}-induced heat shock sensitivity in the presence and absence of *CAC3* overexpression. Again, the heat shock sensitivity and the ability of *CAC3* overexpression to suppress this heat shock sensitivity were not significantly different from the isogenic wild-type strain (Table 2). Thus, *CAC3* overexpression does not appear to affect the *RAS*/cAMP pathway by functioning as a component of a histone modification complex.

***CAC3* suppression of the *RAS*/cAMP pathway is independent of the *GPA2*/*GPR1* signaling pathway.** A parallel pathway for the activation of PKA utilizes the membrane proteins Gpa2p and Gpr1p. Gpa2p is a G_α-like protein which activates adenylyl cyclase in response to extracellular glucose (44). Sch9p is a protein kinase that contributes to the heat shock response independently of the *RAS*/cAMP and *GPA2*/*GPR1* pathways (27). Thevelein and de Winde (44) hypothesized the existence of a *Saccharomyces* protein containing WD40 repeat motifs which could function as a G_β-like protein, suppressing the function of Gpa2p. As *CAC3* contains WD40 repeat motifs, we hypothesized that *CAC3* overexpression might decrease the levels of intracellular cAMP by suppressing the activity of Gpa2p. To test this possibility, we asked if components of the *GPA2* pathway were required for *CAC3*-mediated suppression of *RAS2*^{G19V}-induced heat shock sensitivity. We found that *RAS2*^{G19V} strains lacking *GPA2* or *GPR1* remained sensitive to heat shock and that *CAC3* overexpression effectively suppressed the heat shock sensitivity of these strains (Table 2). Similarly, cells lacking functional *SCH9* were also sensitive to heat shock, and this sensitivity was still suppressed by *CAC3*. Thus, signal transduction through the *GPA2*/*GPR1* or *SCH9* pathway is not required for suppression of *RAS2*^{G19V} by *CAC3* overexpression and Cac3p is not acting as a G_β-like protein to directly suppress Gpa2p.

Identification of Npr1p as a Cac3p-interacting protein. To identify factors that interact with Cac3p and that may be required for *CAC3* suppression of the *RAS*/cAMP pathway, we isolated genes encoding proteins that interact with Cac3p using the yeast two-hybrid system (6). Cac3p was fused to the Gal4p

TABLE. 2. Effect of *CAC3* overexpression on *RAS*/cAMP pathway activation in different mutant strains[a]

Notation type	Strain	Relevant genotype	Heat shock response
RAS/cAMP pathway	YJB195	Wild type	Resistant
	YJB2235	$RAS2^{G19V}$	Sensitive
	YJB2320	$RAS2^{G19V}$ YEp55-*CAC3*	Resistant
	YJB2697	*bcy1*Δ	Sensitive
	YJB2781	*bcy1*Δ YEp55-*CAC3*	Sensitive
Chromatin assembly	YJB2237	$RAS2^{G19V}$ *cac1-1*	Sensitive
	YJB2322	$RAS2^{G19V}$ *cac1-1* YEp55-*CAC3*	Resistant
	YJB3563	$RAS2^{G19V}$ *cac2*Δ	Sensitive
	YJB3876	$RAS2^{G19V}$ *cac2*Δ YEp55-*CAC3*	Resistant
Histone acetyltransferases	YJB2350	$RAS2^{G19V}$ *hat1*Δ	Sensitive
	YJB2367	$RAS2^{G19V}$ *hat1*Δ YEp55-*CAC3*	Resistant
	YJB2648	$RAS2^{G19V}$ *hat2*Δ	Sensitive
	YJB2658	$RAS2^{G19V}$ *hat2*Δ YEp55-*CAC3*	Resistant
	YJB2672	$RAS2^{G19V}$ *hat1*Δ *hat2*Δ	Sensitive
	YJB2673	$RAS2^{G19V}$ *hat1*Δ *hat2*Δ YEp55-*CAC3*	Resistant
	YJB3135	$RAS2^{G19V}$ *gcn5*Δ	Sensitive
	YJB3141	$RAS2^{G19V}$ *gcn5*Δ YEp55-*CAC3*	Resistant
Histone deacetylases	YJB2475	$RAS2^{G19V}$ *hda1*Δ	Sensitive
	YJB2531	$RAS2^{G19V}$ *hda1*Δ YEp55-*CAC3*	Resistant
	YJB2476	$RAS2^{G19V}$ *rpd3*Δ	Sensitive
	YJB2530	$RAS2^{G19V}$ *rpd3*Δ YEp55-*CAC3*	Resistant
	YJB2474	$RAS2^{G19V}$ *had1*Δ*rpd3*Δ	Sensitive
	YJB2532	$RAS2^{G19V}$ *had1*Δ*rpd3*Δ YEp55-*CAC3*	Resistant
Histone transcription	YJB2998	RAS^{G19V} *hir3*Δ	Sensitive
	YJB2999	$RAS2^{G19V}$ *hir3*Δ YEp55-*CAC3*	Resistant
			Resistant
Silencing maintenance	YJB3763	$RAS2^{G19V}$ *sir3*Δ	Sensitive
	YJB3773	$RAS2^{G19V}$ *sir3*Δ YEp55-*CAC3*	Resistant
Cell signaling	YJB3670	$RAS2^{G18A\ G19V}$ *sch9*Δ	Sensitive
	YJB3674	$RAS2^{G18A\ G19V}$ *sch9*Δ YEp55-*CAC3*	Resistant
	YJB5245	$RAS2^{G19V}$ *gpr1*Δ	Sensitive
	YJB5237	$RAS2^{G19V}$ *gpr1*Δ YEp55-*CAC3*	Resistant
	YJB5243	$RAS2^{G19V}$ *gpa2*Δ	Sensitive
	YJB5235	$RAS2^{G19V}$ *gpa2*Δ YEp55-*CAC3*	Resistant

[a] Sensitive indicates that the heat shock response was indistinguishable from that of an isogenic $RAS2^{G19V}$ strain (YJB2235, -2349, -2474, and -3016). Resistant indicates that the heat shock response was indistinguishable from that of an isogenic *RAS2* strain (YJB195, -1583, -2431, and -2136).

DNA-binding domain and used to screen a library of *S. cerevisiae* genes fused to the Gal4p activation domain. One gene identified in this screen, *CAC1*, was subsequently used as a positive control in the screen. Further screening identified a clone containing the codons for amino acids 561 to 605 of *NPR1*. *NPR1* encodes a nitrogen permease reactivator, a putative serine/threonine protein kinase required to regulate the posttranslational stability of several permeases, including Mep2p (26), Gap1p (41), and Tat2p (36). Although the Cac3p-Npr1p interaction is weaker than the Cac3p-Cac1p interaction, it was consistently and reproducibly detected. To biochemically confirm this protein-protein interaction, we constructed strains expressing T7 epitope-tagged Cac3p and either HA-tagged Npr1p or an HA-tagged, kinase-dead version of Npr1p. We immunoprecipitated HA-tagged Npr1 using anti-HA antibodies under nondenaturing conditions, fractionated the immunoprecipitates, and then probed for Npr1p and Cac3p by immunoblotting. As shown in Fig. 3B, Cac3p was coimmunoprecipitated with the putative kinase-dead version of Npr1p, although not with the wild-type version of the protein. This confirms the interaction between Npr1p and Cac3p detected by two-hybrid analysis but suggests that the interaction with the wild-type protein may be relatively transient. Loss of Npr1p kinase activity evidently stabilizes this transient interaction. To the best of our knowledge, neither interaction between Npr1p and the CAF-I complex (or other aspects of chromatin metabolism) nor any connection between Npr1p and the *RAS*/cAMP pathway has been reported previously.

Deletion of *NPR1* yields the same phenotype as does *CAC3* overexpression. To determine if the interaction of Cac3p with Npr1p was responsible for suppression of the *RAS*/cAMP pathway, *NPR1* was deleted in a wild-type or $RAS2^{G19V}$ background, and the resulting strains were assayed for heat shock sensitivity. Deletion of *NPR1* had no affect on the heat shock sensitivity of a wild-type strain but fully suppressed the heat shock sensitivity induced by the $RAS2^{G19V}$ allele (Fig. 4A). In contrast, deletion of *NPR1*, similar to overexpression of *CAC3*, had no effect on the heat shock sensitivity of a *bcy1*Δ strain (Fig. 4B).

Activation of the *RAS*/cAMP pathway causes a sporulation

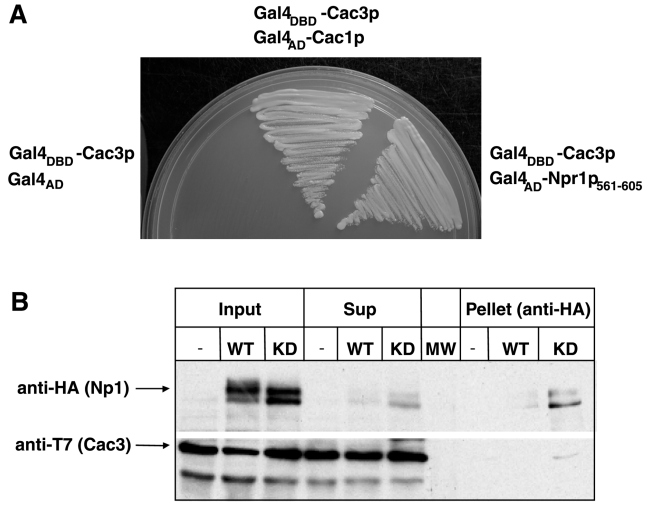

FIG. 3. Cac3p interacts with Npr1p in a two-hybrid assay. (A) $Gal4_{DBD}$-Cac3p/$Gal4_{AD}$ (YJB3523), $Gal4_{DBD}$-Cac3p/$Gal4_{AD}$-Cac1p (YJB3197), and $Gal4_{DBD}$-Cac3p/$Gal4_{AD}$-$Npr1p_{561–605}$ (YJB3438) strains carrying the *HIS3* gene under control of the *GAL1* promoter were plated on SDC lacking Leu, Trp, and His and with 5 mM 3AT. Growth indicates a positive interaction between the Gal4 DNA-binding domain (DBD) and Gal4 activation domain (AD) fusion proteins. (B) Cac3p coimmunoprecipitates with Npr1p. Yeast strains overexpressing T7 epitope-tagged Cac3p either without (lane —) or with the coover-expression of HA epitope-tagged wild-type Npr1p (WT) or kinase-dead Npr1p (KD) were immunoprecipitated under native conditions with anti-HA antiserum. Levels of Npr1p and Cac3p were determined from the input, supernatants (sup), and pellets by immunoblotting with either anti-HA or anti-T7 antiserum. Lane MW, size markers.

defect (21), which can be suppressed by overexpression of *CAC3* (35) (Table 3). Similar to *CAC3* overexpression, deletion of both copies of *NPR1* suppressed the sporulation defect caused by the $RAS2^{G19V}$ allele (Table 3). The fact that *CAC3* overexpression or *NPR1* deletion can suppress two distinctly different phenotypes of an activated *RAS*/cAMP pathway indicates that this effect is specific to this pathway and does not reflect a generalized increase in heat tolerance. Thus, the phenotypes of strains lacking *NPR1* are indistinguishable from the phenotypes of strains overexpressing *CAC3*: both mutations suppress the *RAS*/cAMP signal transduction pathway when it is activated by a $RAS2^{G19V}$ allele but not by *BCY1* deletion.

These data suggest a model in which excess Cac3p interacts with Npr1p, sequestering the kinase so that it is not available for its role in the *RAS*/cAMP pathway. The sequestration model predicts that (i) *CAC3* overexpression in an *npr1*Δ strain would confer no additional resistance to heat shock, (ii) the ability of an *npr1*Δ mutation to suppress $RAS2^{G19V}$ phenotypes would not depend on the presence of a functional *CAC3* gene, and (iii) elevated levels of *NPR1* would provide excess copies of Npr1p and negate the effect of *CAC3* overexpression. Consistent with the first prediction, no additional heat shock resistance was observed when *CAC3* was overexpressed in an *npr1*Δ strain (Fig. 4A), which is consistent with the idea that *CAC3* overexpression and *NPR1* deletion are suppressing the *RAS*/cAMP pathway by a common mechanism. Consistent with the second prediction, $RAS2^{G19V}$ cells carrying deletions of both *NPR1* and *CAC3* remained heat shock resistant like the $RAS2^{G19V}$ *npr1*Δ cells (data not shown). Consistent with the

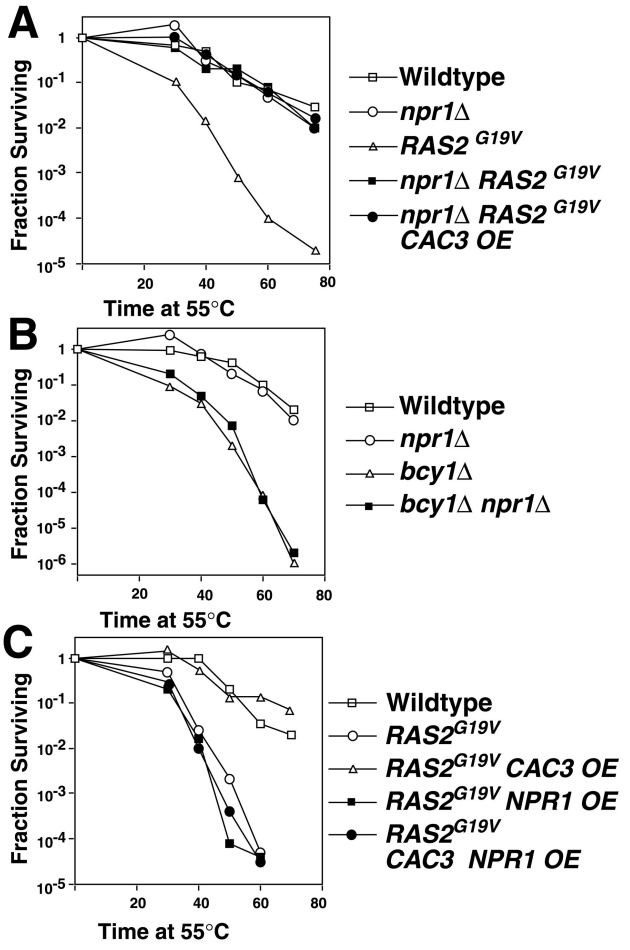

FIG. 4. Deletion of *NPR1* suppresses the *RAS*/cAMP pathway between RAS and PKA. The heat shock resistance of (A) wild-type (YJB195), *npr1*Δ (YJB3712), $RAS2^{G19V}$ (YJB2235), $RAS2^{G19V}$ *npr1*Δ (YJB2723), and $RAS2^{G19V}$ *npr1*Δ *CAC3*-overexpressing (OE) (YJB4017), and (B) *bcy1*Δ (YJB2697) and *bcy1*Δ *npr1*Δ (YJB3554) strains was determined. (C) The heat shock resistance of isogenic wild-type (YJB195), $RAS2^{G19V}$ (YJB2235), and $RAS2^{G19V}$ strains carrying high-copy *CAC3* (YJB2320), *NPR1* (YJB3863) and both *CAC3* and *NPR1* (YJB3875) was determined.

third prediction, $RAS2^{G19V}$ cells remained sensitive to heat shock when both *CAC3* and *NPR1* were overexpressed from the *GAL1* promoter (Fig. 4C).

In addition, the sequestration model predicts that if excess Cac3p sequesters Npr1p, then the phenotypes of cells overex-

TABLE. 3. Deletion of *NPR1*, like overexpression of *CAC3*, suppresses the sporulation defect of $RAS2^{G19V}$ strains

Strain	Relevant genotype	Sporulation efficiency (%)
YJB334	Wild type	14.9
YJB3737	$RAS2^{G19V}$	4.8
YJB3792	*npr1*Δ/*npr1*Δ	23.0
YJB3791	$RAS2^{G19V}$ *npr1*Δ/*npr1*Δ	18.1
YJB3739	YEp55-CAC3	47.2
YJB3721	$RAS2^{G19V}$ YEp55-*CAC3*	34.0
YJB5523	$RAS2^{G19V}$ pCUP1-myc-UBI4	18.1

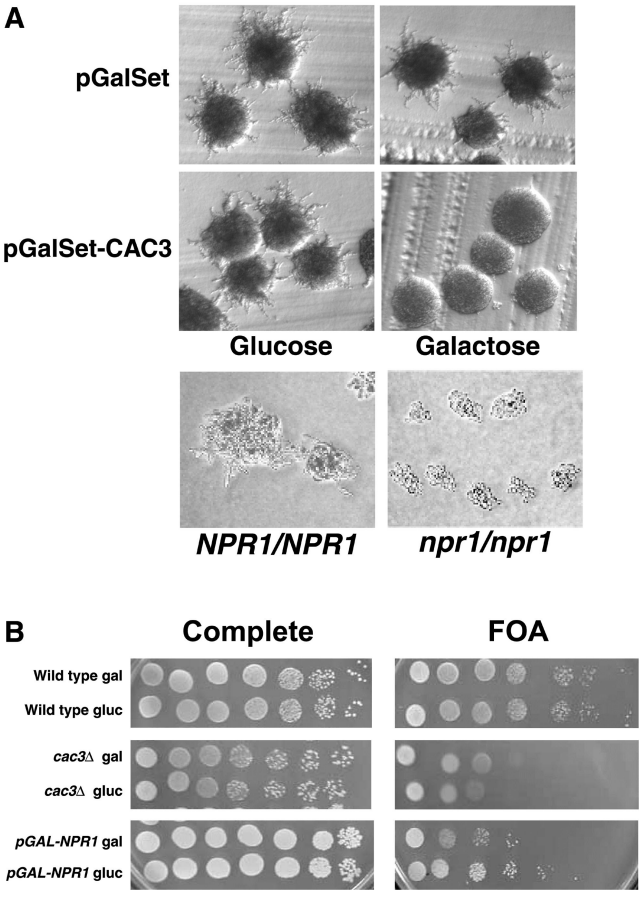

FIG. 5. *CAC3* and *NPR1* are mutally antagonistic. (A) *CAC3* overexpression suppresses pseudohyphal growth. Σ1278B strains carrying either pGalSet (YJB3514) or pGalSet-*CAC3* (YJB3515) were grown in either galactose (to induce *CAC3* expression) or glucose (to repress *CAC3* expression). The cells were then plated on either SLAD or SLADG medium to induce pseudohyphal growth either with or without *CAC3* expression. Representative colonies were photographed after 3 days of growth at 30°C. For comparison, wild-type (YJB5724) and *npr1/npr1* (YJB5723) Σ1278B strains are also shown. (B) *NPR1* overexpression decreases telomeric silencing. Serial dilutions (1:10) of wild-type (YJB3610), *cac3* (YJB1786), and pGalSET-*NPR1* (YSJ328) strains were plated on either complete medium or medium with 5-fluoroorotic acid (FOA). Yeast cells were grown in medium containing either glucose (gluc) or galactose (gal), as indicated. Plates were photographed after 2 days of growth at 30°C.

pressing *CAC3* should resemble the known phenotypes of cells lacking *NPR1*. Npr1p is required for the stable expression of the Mep2p ammonium transporter, which is necessary for pseudohyphal growth in diploid strains in the Σ1278b strain background. Homozygous deletion of *NPR1* in the diploid Σ1278b strain background results in cells that produce fewer pseudohyphae (Fig. 5A, bottom panels), apparently due to the loss of Mep2p function (26). As predicted by the sequestration model, overexpression of *CAC3* in a diploid Σ1278b strain, like deletion of *NPR1* in these strains, greatly reduced the number and length of pseudohyphae formed (Fig. 5A, upper panels). Thus, *CAC3* overexpression and *NPR1* deletion both result in identical phenotypes for suppression of the *RAS*/cAMP pathway and for pseudohyphal growth.

Furthermore, the sequestration model predicts that overexpression of *NPR1* should produce the same phenotypes as deletion of *CAC3*. Kaufman et al. (24) showed that deletion of *CAC3* leads to a decrease in transcriptional silencing of a *URA3* reporter gene at the telomere on chromosome VII by about 25-fold. The *NPR1* gene was placed on a plasmid under control of the *GAL1, 10* promoter to be induced in the presence of galactose and repressed in the presence of glucose. *NPR1*-overexpressing cells, like *cac3*Δ cells, have a modest decrease in telomeric silencing of approximately 16-fold (Fig. 5B). Taken together, these data are consistent with our proposed model that excess Cac3p binds to Npr1p and sequesters the kinase, resulting in a set of phenotypes that are indistinguishable from those caused by *npr1*Δ.

Npr1p does not affect the phosphorylation state or localization of Ras2p. Ras2p is a membrane-associated phosphoprotein whose activity is increased by phosphorylation of Ser-214 (50) by an unknown kinase. Npr1p is a putative serine/threonine kinase that presumably phosphorylates several membrane proteins (26, 36, 41). To test the hypothesis that Npr1p may be the kinase that phosphorylates Ras2p, we metabolically labeled wild-type and *npr1*Δ cells with ^{32}P, immunoprecipitated Ras2p, performed SDS-PAGE, and detected phospho-Ras2p by autoradiography. The amount and electrophoretic mobility of phospho-Ras2p were not affected by deletion of *NPR1* (Fig. 6A), indicating that Npr1p does not affect the degree of Ras2p phosphorylation either directly or indirectly. Immunoblot experiments did not detect any change in the quantity of total Ras2p in $RAS2^{G19V}$ cells overexpressing Cac3p or missing Npr1p (data not shown). Furthermore, recombinant Npr1p did not phosphorylate recombinant human Ha-Ras (data not shown). Thus, it appears unlikely that Npr1p modulates the *RAS*/cAMP pathway by affecting the phosphorylation of Ras. We cannot rule out the formal possibility that Npr1p is one of several kinases that can phosphorylate Ras in vivo, although this scenario cannot explain how loss of Npr1p function alone could suppress the *RAS*/cAMP pathway while not causing any detectable change in the degree of Ras phosphorylation.

In order to function correctly, Ras2p must localize to the plasma membrane. Mislocalization of Ras2p decreases its signaling activity and effectively suppresses the constitutively active $RAS2^{G19V}$ allele (2, 4). Thus, we asked if deletion of *NPR1* or overexpression of *CAC3* altered the membrane localization of Ras2p. When the *RAS2* product is fused to GFP, the plasma membranes of yeast cells fluoresce, indicating that Ras2p is located at the membrane (4) (Fig. 6B). Deletion of *NPR1* or overexpression of *CAC3* did not alter the peripheral localization pattern of Ras2p-GFP (Fig. 6B), suggesting that suppression of the $RAS2^{G19V}$ allele by excess Cac3p is not due to an alteration in the subcellular distribution of Ras2p.

CAC3 overexpression and *npr1*Δ lead to indistinguishable phenotypes, leading us to propose a model in which Cac3p binds and sequesters Npr1p. Another possibility is that overexpression of *CAC3* decreases the quantity of Npr1p, perhaps by targeting Npr1p for destruction. To test this possibility, we used a series of yeast strains containing an epitope-tagged *NPR1* gene (36). The overexpression of *CAC3* in either the presence or absence of the $RAS2^{G19V}$ allele had no effect on the amount of Npr1p present in the cell (data not shown). Thus, consistent with the sequestration model, excess copies of

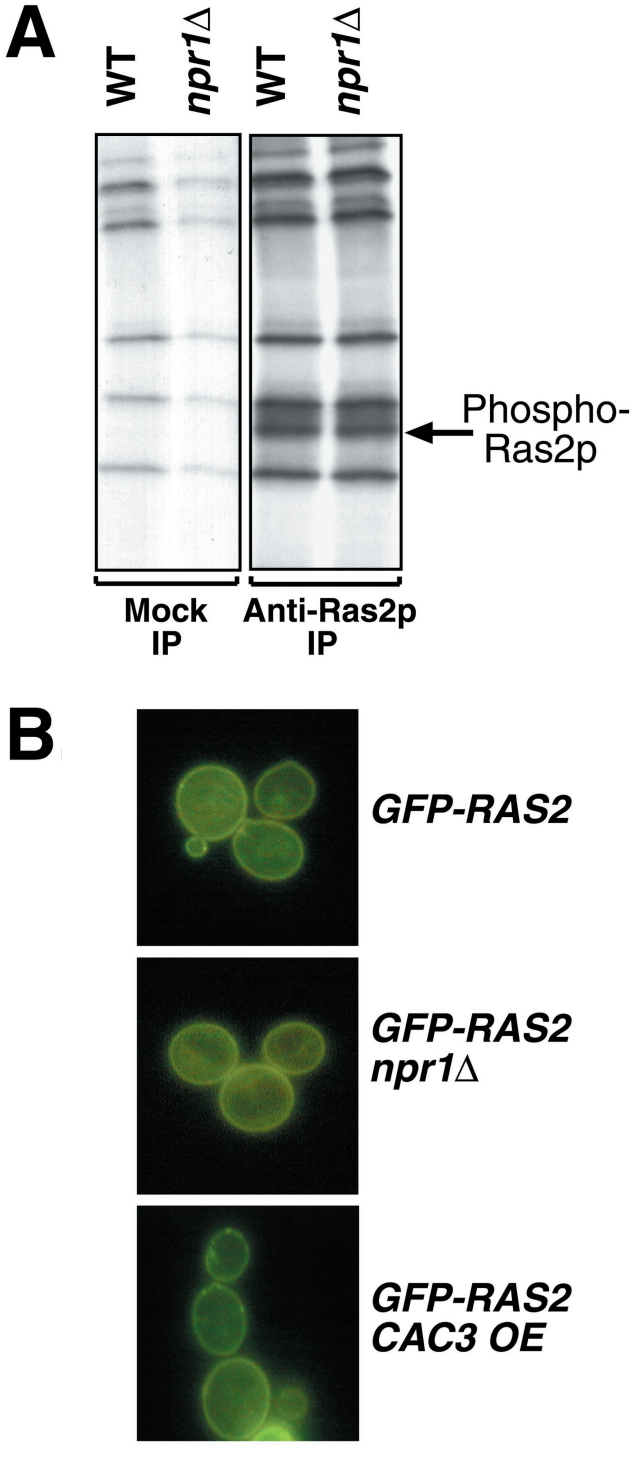

FIG. 6. Npr1p does not affect the phosphorylation or localization of Ras2p. (A) Wild-type (WT) (YJB195) and *npr1*Δ (YJB3712) cells were grown in the presence of [^{32}P]orthophosphate. Lysates were immunoprecipitated (IP) with protein A-agarose (mock) or anti-Ras2p conjugated to agarose. Bound proteins were eluted by denaturation, separated by SDS-PAGE, and detected by autoradiography. The arrow indicates phospho-Ras2p. (B) Localization of GFP-Ras2p was determined by fluorescence microscopy in wild-type (YJB3896) and *npr1*Δ (YJB3897) strains and cells expressing extra copies of *CAC3* (YJB3898).

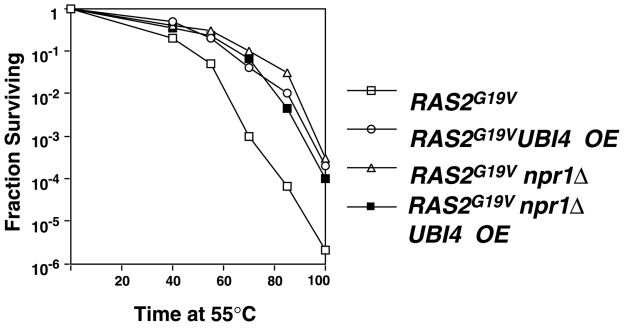

FIG. 7. Overexpression of polyubiquitin (*UBI4*) suppresses the *RAS2*^{G19V}-induced heat shock sensitivity. The heat shock resistance of *RAS2*^{G19V} (YJB2235), *RAS2*^{G19V} *UBI4*-overexpressing (OE) (YJB27334), *RAS2*^{G19V} *npr1*Δ (YJB3723), and *RAS2*^{G19V} *npr1 UBI4*-overexpressing (YJB5522) strains in the presence and absence of *NPR1* and *UB14* overexpression were determined as for Fig. 1.

Cac3p did not destabilize Npr1p, yet still caused the same phenotypes as in an *npr1*Δ strain.

Ubiquitin overexpression can suppress the *RAS*/cAMP pathway. Npr1p is thought to stabilize Mep2p and other permeases by phosphorylating the protein, which then reduces the protein's ubiquitination and subsequent inactivation (26, 41). We hypothesized that, in a similar manner, Npr1p may stabilize another substrate protein that is important for the full function of the *RAS*/cAMP pathway by inhibiting its ubiquitination. Thus, deletion of *NPR1* would be expected to increase the ubiquitination of putative substrate proteins. Furthermore, if this hypothesis is correct, overexpression of *UBI4*, the gene encoding polyubiquitin, would be expected to increase the ubiquitination of this substrate protein. Consistent with this expectation, overexpression of Myc epitope-tagged *UBI4* from the copper-inducible *CUP1* promoter on a high-copy plasmid suppressed *RAS2*^{G19V}-induced heat shock sensitivity (Fig. 7). Additionally, overexpression of *UBI4* in a *RAS2*^{G19V} *npr1*Δ strain conferred no additional heat shock resistance, suggesting that these two genes suppress the *RAS*/cAMP signal transduction pathway by a common mechanism. Furthermore, overexpression of *UBI4* suppressed the sporulation defect of *RAS2*^{G19V} cells (Table 3). Thus, overexpression of polyubiquitin suppressed activated *RAS2* phenotypes in a manner indistinguishable from either *CAC3* overexpression or *NPR1* deletion. This result is consistent with the hypothesis that *NPR1* and ubiquitin have antagonistic roles in the suppression of the *RAS*/cAMP pathway.

DISCUSSION

Cac3p has at least two separable functions. Cac3p is the smallest subunit of CAF-I, which assemble histones H3 and H4 tetramers onto newly replicated DNA (24, 38). In addition, excess Cac3p suppresses the activated *RAS*/cAMP pathway (35) (Fig. 1) in a CAF-I independent manner (Fig. 2), a result consistent with a recent report (51). Because *CAC3* acts antagonistically with the Sin3p-Rpd3p complex (42) and the mammalian Cac3p homolog (RbAp48) is associated with chromatin-modifying enzymes such as histone deacetylases and histone acetylases (43, 49), we asked if the *CAC3* suppression of *RAS2*^{G19V} required histone-modifying activities such as the

histone deacetylases Rpd3p and Hda1p or the histone acetyltransferases Hat1p, Hat2p, and Gcn5p. Interestingly, we found that *CAC3* suppression of $RAS2^{G19V}$ was not dependent upon any of the histone deacetylases or histone acetylases tested, nor was it dependent upon the histone regulator Hir3p or the silent chromatin component Sir3p (Table 2). Furthermore, several histone deacetylase complexes, including those containing Rpd3p and Hda1p, have been characterized in biochemical fractionation studies, and Cac3p has not been isolated as a component of these complexes (M. Grunstein, personal communication). Therefore, we conclude that the role of Cac3p in the *RAS*/cAMP signal transduction pathway is distinct and separable from the role of Cac3p in CAF-I-dependent chromatin assembly and histone modification. Consistent with this conclusion, *CAC3* mRNA does not share the cell cycle-dependent expression pattern identified for *CAC1* and *CAC2* (39), not all Cac3p/p48 copurifies with the CAF-I complex (28, 32, 43), and the subcellular localization of Cac3p is distinct from that of Cac1p (Fig. 2C).

Cac3p suppression of the *RAS*/cAMP pathway is mediated by Npr1p. A C-terminal fragment of Npr1p, which includes a portion of the kinase domain, interacted with Cac3p in a yeast two-hybrid screen and biochemically (Fig. 3). Consistent with the hypothesis that Cac3p overexpression reduces the effective activity of Npr1p, the suppression phenotypes of *npr1*Δ strains (e.g., heat shock resistance, sporulation, and loss of pseudohyphal growth) were indistinguishable from the phenotypes of strains overexpressing *CAC3* (Fig. 4 and 5). Both mutations were capable of suppressing two different phenotypes caused by the $RAS2^{G19V}$ allele but not the same phenotypes resulting from deletion of the negative regulator *BCY1*. This demonstrates that overexpression of *CAC3* and deletion of *NPR1* suppress the pathway in an indistinguishable manner, suggesting a common suppression mechanism which is dependent on the *RAS*/cAMP pathway. Furthermore, overexpression of *CAC3* in an *npr1*Δ strain did not enhance the suppression phenotype, supporting the idea that Cac3p affects the *RAS*/cAMP pathway by reducing the level of active, available Npr1p. Finally, cooverexpression of *NPR1* blocked the ability of *CAC3* overexpression to reduce the heat shock sensitivity of a $RAS2^{G19V}$ strain (Fig. 4C), supporting the model that excess Cac3p binds and sequesters Npr1p.

NPR1 encodes a nitrogen permease reactivator, a putative serine/threonine kinase that affects the activity of several nutrient transporters (13, 14), including Gap1p, the general amino acid permease (46); Mep2p, an ammonium permease (26); Pcp1p, a spermidine transporter (19); and Tat2p, the tryptophan transporter (36). *NPI1*/*RSP5*, encoding a ubiquitin-protein ligase, antagonizes the activity of *NPR1* in many cases (14, 17). The currrent model postulates that phosphorylation of a transporter by Npr1p affects ubiquitination and subsequent proteolysis of that transporter, stabilizing nutrient-repressible permeases such as Gap1p (40, 41) but promoting degradation of constitutive permeases such as Tat2p (36). In its interaction with the *RAS*/cAMP pathway, *NPR1* acts antagonistically with ubiquitin. We found that overexpression of polyubiquitin suppressed $RAS2^{G19V}$-induced heat shock sensitivity in a manner that was indistinguishable from the suppression observed in *npr1*Δ strains (Fig. 7). The target of rapamycin (TOR) nutrient signaling pathway leads to the phosphorylation and subsequent inhibition of Npr1p (36). For more than 15 years, we have known that *NPR1* is a key regulator of nitrogen metabolism (14) and that *RAS*/cAMP pathway is the principal regulator of carbon metabolism in *Saccharomyces* (5). This work is the first reported example of cross-talk between these two metabolic regulatory pathways. We have demonstrated that Npr1p affects the activity of the *RAS*/cAMP signal transduction pathway, providing a heretofore unrecognized connection between the carbon and nitrogen signaling pathways.

The precise mechanism by which Npr1p affects the *RAS*/cAMP pathway remains unknown. Neither the *TOR1-1* or *TOR2-1* mutation nor treatment with rapamycin had any effect on the ability of either *CAC3* overexpression or *NPR1* deletion to suppress the $RAS2^{G19V}$ phenotype (data not shown). This indicates that the TOR-dependent phosphorylation state of Npr1p does not affect the role of Npr1p in *RAS*/cAMP signaling. Since Npr1p affects the stability of a number of proteins and since overexpression of polyubiquitin yields the same phenotype as loss of Npr1p, we surmise that Npr1p activates or potentiates the *RAS*/cAMP pathway by stabilizing one or more intermediates in the pathway. Furthermore, the fact that overexpression of *CAC3* or deletion of *NPR1* suppresses $RAS2^{G19V}$-induced phenotypes but not the same phenotypes resulting from deletion of *BCY1* suggests that Npr1p acts between Ras-induced synthesis of cAMP and cAMP-mediated activation of the A kinase. Deletion of *NPR1* does not affect the phosphorylation, ubiquitination, localization, or abundance of Ras2p (Fig. 6 and data not shown), nor does deletion of *NPR1* affect the levels of Cdc25p, the guanine nucleotide exchanger for Ras (L. Schneper and J. R. Broach, unpublished observations).

Zhu and colleagues (51) recently reported that *CAC3* overexpression suppressed the *RAS*/cAMP pathway when that pathway was activated by deletion of *PDE1* and *PDE2* or by an activated *TPK2* mutation. However, *CAC3* overexpression did not affect the total level of extractable PKA kinase activity. Their data indicated that *CAC3* suppressed the *RAS*/cAMP pathway in a *CAC1*-independent and *BCY1*-dependent fashion which is not fully understood (51). The results presented here support and extend their conclusions by indicating that Npr1p is the target of Cac3p that modulates the *RAS*/cAMP signal transduction pathway.

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