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PP2A Phosphatase Activity Is Required for Stress and Tor Kinase Regulation of Yeast Stress Response Factor Msn2p

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In response to stress and nutrient starvation, the *Saccharomyces cerevisiae* transcription factor Msn2p accumulates in the nucleus and activates expression of a broad array of genes. Here, we analyze the role of the Tor (target of rapamycin) signaling pathway in mediating these responses. Inactivation of the Tor pathway component Tap42p using *tap42*(Ts) alleles causes a sustained nuclear localization similar to that after the addition of the Tor kinase inhibitor rapamycin. Effects of Tap42p inactivation and rapamycin addition could be suppressed by deletion of *TIP41*, which encodes a Tap42p-interacting protein. These results support the notion that rapamycin affects Msn2p by inactivating Tap42p function. Tap42p interacts with the catalytic subunit of PP2A (protein phosphatase 2A) and PP2A-like phosphatases. Deletion of either the catalytic or regulatory subunit that forms the PP2A phosphatase complex prevents nuclear accumulation of Msn2p in the *tap42*(Ts) strain and in wild-type strains treated with rapamycin. These results suggest that Tap42p is an inhibitor of PP2A phosphatase, which in turn inhibits nuclear export of Msn2p. Interestingly, PP2A function is also required for nuclear accumulation of Msn2p in response to stresses, such as heat and osmotic shock, as well as nitrogen (but not glucose) starvation. Thus, PP2A and the Tor kinase pathway transduce stress and nitrogen starvation signals to Msn2p. Finally, Msn2p localization is unaffected by conditional loss of 14-3-3 protein function, ruling out the possibility that 14-3-3 proteins act as a scaffold to sequester Msn2p in the cytoplasm.

Eukaryotic cells, such as the yeast *Saccharomyces cerevisiae*, must sense and respond to a broad array of chemical and physical insults. *S. cerevisiae* cells have developed specialized signal transduction pathways to cope with these insults; however, they also harbor a general stress response pathway that can be stimulated by seemingly unrelated stress conditions (heat and osmotic shock, UV, oxidative stress, and nutrient depletion) and that enables them to survive aberrant protein folding, DNA damage, oxidant accumulation, and starvation. The functionally redundant transcription factors Msn2p and Msn4p are key players in the general stress response pathway. During mitotic growth, Msn2p and Msn4p reside in the cytoplasm where they are inactive. Upon exposure to stress or nutrient depletion, these transcription factors rapidly accumulate in the nucleus and stimulate expression of more than 150 genes (15, 16, 36, 39), which provide transient protection against further insult from both the same and different stresses.

At least two *cis*-acting domains function together to regulate Msn2p localization. A nuclear localization signal (NLS) within the carboxyl-terminal half of Msn2p responds to glucose levels but not to stress, so that glucose starvation results in rapid nuclear accumulation of a reporter protein containing the Msn2p NLS and a constitutive export signal (15, 16). In contrast, a second domain within the amino terminus of Msn2p responds to both of these stimuli. The localization function of this domain is less defined than that of the carboxyl-terminal NLS; however, it is thought to involve nuclear export. This conclusion was based on two observations. First, regulated localization of the amino-terminal domain requires an exogenous NLS, and second, the localization of this region is sensitive to mutations (*msn5*Δ) in the nuclear export machinery (16). The sensitivity of the amino-terminal domain to both stress and glucose depletion implies that it must contain either a single nuclear export signal (NES) that can be coregulated by glucose and stress or two separate subdomains that can be independently regulated by glucose and stress (16).

The first signaling pathway implicated in Msn2p localization was the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway (15). PKA inactivation results in rapid nuclear accumulation of Msn2p (15); however, the pathway appears to transmit only the glucose signal, because stress does not affect the PKA-responsive carboxyl-terminal NLS. Moreover, glucose starvation, but not stress, is associated with a rapid drop in phosphorylation of several PKA-sensitive serines within the NLS (16). Thus, PKA phosphorylates and inactivates the NLS until it is unmasked by glucose depletion. Many mechanisms could explain how phosphorylation contributes to cytoplasmic retention of Msn2p; however, Beck and Hall (2) recently proposed a model in which the yeast 14-3-3 protein homologs, Bmh1p and Bmh2p, interact with and sequester phosphorylated Msn2p in the cytoplasm. Their model was based on the observation that the biochemical interaction between Bmh1p/Bmh2p and Msn2p proteins was disrupted by conditions that

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cause Msn2p to accumulate in the nucleus (2), as well as a generally accepted role for mammalian 14-3-3 proteins in sequestering mammalian signaling proteins. The model proposed by Beck and Hall (2) was given further support by previous suggestions that Bmh1p and Bmh2p have a functional, if poorly understood, effect on PKA-regulated processes, such as growth and pseudohyphal formation (13, 31).

Identification of the potential stress response pathway stems from the observation that inactivation of the Tor (Target of rapamycin) kinase pathway caused Msn2p to accumulate in the nucleus (2, 16, 29). Tor kinase is a phosphatidylinositol kinase-related protein kinase that was originally identified by the isolation of mutations (*TOR1-1*) that confer resistance to the growth inhibitory properties of the immunosuppressive drug rapamycin. Rapamycin forms a toxic complex with its intracellular receptor FKBP12, which then binds and inhibits Tor kinase (20, 27). The Tor kinase pathway plays an important, if incompletely understood, role in controlling growth in response to nutrients, because cells depleted of Tor kinase activity (by rapamycin addition or Tor inactivation) exhibit physiological characteristics of starved or stressed cells, including G_1 arrest, a decrease in macromolecular synthesis, and induction of general stress response genes (1, 8). A role for the Tor kinase pathway in stress response was supported by the observation that Tor inactivation (rapamycin treatment) impinged only on the stress-sensitive amino-terminal domain of Msn2p (16).

Several downstream effectors of Tor kinase have been identified (7). The effector that is most relevant to the general stress response pathway is Tap42p, an essential protein that interacts with catalytic subunits of the type 2A (and 2A-like) protein phosphatases (8). Nitrogen starvation and Tor kinase inactivation result in Tap42p dephosphorylation and subsequent dissociation of Tap42-PP2A (protein phosphatase 2A) and Tap42-PP2A-like phosphatase complexes (8, 24). Yeast PP2A phosphatase is a heterotrimer that is comprised of a catalytic subunit (subunit C), regulatory subunit (subunit B), and a scaffolding subunit (subunit A). Two homologous genes, *PPH21* and *PPH22*, redundantly encode the PP2A catalytic subunit, the loss of which results in retarded growth, defects in actin organization, and difficulty progressing through mitosis (26, 33). Regulatory subunit activity is also encoded by two genes, *CDC55* and *RTS1*, although in this case the two proteins are thought to perform different cellular functions, targeting the phosphatase complex to distinct substrates and cellular processes (14, 37, 45). Finally, the scaffolding subunit is encoded by a single gene, *TPD3* (44). *S. cerevisiae* also contains a PP2A-like phosphatase activity which is comprised of the catalytic subunit Sit4p and several regulatory subunits (8, 28). Although there is no general consensus as to whether Tap42p stimulates or inhibits phosphatase activity, recent results are consistent with a model in which Tap42p inhibits PP2A and PP2A-like phosphatase activity in at least some of its functions (2, 6, 35).

We recently used a *tap42*(Ts) mutation to show that Tap42p inactivation, like rapamycin treatment, caused Msn2p to accumulate in the nucleus (9). To examine whether the effects of rapamycin treatment are mediated by Tap42, we monitored Msn2p localization and function in strains lacking components of the Tor/Tap42/PP2A phosphatase pathway. We provide evidence to support the hypothesis that rapamycin effects on Msn2p are the result of Tap42p inactivation and show that PP2A, but not PP2A-like, catalytic activity is necessary for Msn2p nuclear accumulation and transcriptional activation in response to rapamycin treatment. Interestingly, PP2A activity is also necessary for stress-induced translocation. These results suggest that Tap42p functions in the general stress response pathway to inhibit or sequester PP2A phosphatase. In response to stress or rapamycin, Tap42 inhibition of PP2A is relieved, and dephosphorylation of Msn2p (or an Msn2p-interacting protein) stimulates Msn2p accumulation in the nucleus.

MATERIALS AND METHODS

Media and growth conditions. Cells were grown in rich (yeast extract-peptone-dextrose [YEPD]) or synthetic complete (SC) medium lacking the appropriate amino acids (25). Temperature shifts were achieved by resuspending harvested cells into prewarmed medium. Rapamycin (Sigma) was added to a concentration of 200 ng/ml as described previously (9). Medium containing 1 M sorbitol or lacking glucose has been described elsewhere (16). Nitrogen starvation medium was made by using yeast nitrogen base lacking ammonium sulfate and amino acids.

***S. cerevisiae* strains.** *S. cerevisiae* strains used in the study are listed in Table 1.

Plasmids. The integrating Msn2-myc$_{12}$ plasmid pAK3-3, full-length Msn2-green fluorescent protein (GFP) plasmid p*ADH-MSN2-GFP*, and the *put1-lacZ* expression reporter plasmid pWB36 have been described previously (7, 16, 34). The *BMH1* plasmid pAL110 consists of a 3-kb DNA fragment, which extends from 400 bp upstream of *BMH1* to 600 bp past the downstream gene *PDA1*, inserted into the low-copy-number *LEU2 CEN* plasmid pRS315. Plasmid pAL99 was constructed by inserting a 2-kb HincII fragment containing *BMH1* and *PDA1* into the same site of pBSKII. Plasmid pAL100 is a *pda1*::*TRP1* derivative of pAL99 and was constructed by inserting a Klenow fragment-treated EcoRI-BglII *TRP1* fragment into the Klenow fragment-treated NcoI site within *PDA1*. The *CTT1-lacZ* fusion plasmid pAL22 was constructed by inserting a 9.2-kb HindIII fragment containing *CTT1-lacZ* embedded in *URA3* (38) into the *LEU2 CEN* vector pSB32 (40).

Isolation of the *bmh1-11*(Ts) *bmh2*::*LEU2* mutant. A conditional allele of *BMH1* was identified by replacing the *BMH1* gene of *BMH1 bmh2*::*URA3* strain SL1320 with hydroxylamine-mutagenized *BMH1-pda1*::*TRP1* DNA (pAL100) and screening Trp$^+$ colonies for growth at 23 and 36°C. Tetrad analyses showed that the temperature-sensitive growth defect of one mutant (AHY316) was tightly linked to the *pda1*::*TRP1* marker, dependent upon the presence of the *bmh2*::*URA3* disruption, and complemented by transformation with low-copy-number *BMH1* plasmid pAL110. Moreover, sequence analysis showed that the temperature-sensitive *bmh1* allele designated *bmh1-11*(Ts) was the result of a G-to-A substitution at codon 193 that converted an invariant Ala to Thr. This contrasts with the previously described *bmh1* temperature-sensitive allele with a S189P substitution (43). Despite being implicated in several growth-related signaling pathways (12, 13, 31, 43), the only terminal phenotype associated with 14-3-3 protein dysfunction of *bmh1-11*(Ts) *bmh2*::*URA3* haploid strain SGY459 was a slight (40 to 65%) increase in large budded cells. This increase was not associated with either an increase in cells with 2*N* DNA or discrete nuclear morphology (data not shown).

β-Galactosidase activity. β-Galactosidase activity was measured by the permeabilized cell method (25) and are expressed as $(1{,}000 \times OD_{420})/(OD_{600} \times$ volume [assayed in milliliters] $\times$ time [in minutes]), where OD_{420} and OD_{600} are the optical densities at 420 and 600 nm, respectively.

Fluorescence microscopy. Cells expressing Msn2-myc$_{12}$ or Msn2-GFP were grown to mid-log phase before treatment. For immunofluorescence, cells were fixed in 3.7% formaldehyde and spheroplasts were prepared (32). Msn2-myc$_{12}$ was visualized using 9E10 monoclonal anti-myc antibody purchased from Covance (Richmond, Calif.). The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody from Roche Diagnostics (Indianapolis, Ind.). DNA was visualized by staining with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) from Sigma-Aldrich (St. Louis, Mo.) as described previously (9). Msn2-GFP was visualized directly after incubating cells for the final 15 min in medium containing Hoechst 33258 stain from Sigma-Aldrich.

TABLE 1. Strains of *S. cerevisiae* used in this study

Strain	Relevant genotype	Reference or source
SGY446	*MAT*α *ura3-52 his3 leu2-3,112 trp1 ade8 tpk1*::*ADE8 tpk2-63*(Ts) *tpk3*::*TRP1*	39
AKY54	SGY446 (YEp352-*BMH2*)	This study
AKY53	SGY446 (YEp352)	This study
AKY31	SGY446 *TPK2* (YEp352)	This study
AHY148	*MAT***a** *ura3-52 his3 leu2-3,112 trp1 ade8 tpk1*^{w1} *tpk2*::*HIS3 tpk3*::*TRP1*	18
RTF1.5-2	*MAT*α *ura3-52 his3 leu2-3,112 trp1 ade8 tpk1*^{w2} *tpk2*::*HIS3 tpk3*::*TRP1 bcy1*::*URA3*	5
AHY240a	*MAT***a** *ura3-52 his3 leu2-3,112 trp1 ade8 TPK1 tpk2*::*HIS3 tpk3*::*TRP1 bmh1*::*ADE8*	This study
AHT240b	*MAT***a** *ura3-52 his3 leu2-3,112 trp1 ade8 tpk1*^{w1} *tpk2*::*HIS3 tpk3*::*TRP1 bmh1*::*ADE8*	This study
SL1320	*MAT*α *ura3 leu2 his3 BMH1 bmh2*::*URA3*	13
SGY459	*MAT*α *ura3 leu2 his3 pda1*::*TRP1 bmh1-11*(Ts) *bmh2*::*URA3*	This study
AKY1	SGY459(pRS315)	This study
AKY2	SGY459(pAL110)	This study
AKY24	SGY459 *msn2*::*HIS3*	This study
AKY25	SGY459 *msn2*::*HIS3 msn4*::*LEU2*	This study
AKY5	*MAT*α *ura3 leu2 his3 bmh1-11*(Ts) *bmh2*::*KanMX*	This study
AKY9	AKY5(pRS315)	This study
AKY10	AKY5(pAL110)	This study
AKY44	AKY5 (YEp352)	This study
AKY45	AKY5 (YEp352-*TPK2*)	This study
AKY46	AKY5 (YEp352-*TPK3*)	This study
AKY32	AKY5 *MSN2*::pRS306-*MSN2-myc*$_{12}$(pAL110)	This study
AKY33	AKY5 *MSN2*::pRS306-*MSN2-myc*$_{12}$(pRS315)	This study
AKY35	SGY559(pWB36)	This study
Y3033	*MAT***a** *tap42*::*HIS3*(pRS414-*TAP42*)	9
Y3036	Y3033 *msn2*::*LEU2 msn4*::*URA3*	This study
Y3034	*MAT***a** *tap42*::*HIS3* [pRS414-*tap42-106*]	9
Y3037	Y3034 *msn2*::*LEU2 msn4*::*URA3*	This study
Y3035	*MAT***a** *tap42*::*HIS3* [pRS414-*tap42-106*]	9
Y3038	Y3035 *msn2*::*LEU2 msn4*::*URA3*	This study
AKY38	Y3033 *MSN2*::pRS306-*MSN2-myc*$_{12}$	9
AKY79	Y3033 p*ADH-MSN2-GFP*	This study
AKY84	Y3034 p*ADH-MSN2-GFP*	This study
AKY75	AKY38 *tip41*::*KanMX*	This study
AKY142	AKY79 *tip41*::*KanMX*	This study
AKY144	AKY84 *tip41*::*KanMX*	This study
AKY148	*MAT***a** *tap42*::*HIS3*(pRS414-*TAP42*)(pAL22)	This study
AKY150	*MAT***a** *tap42*::*HIS3*(pRS414-*tap42-106*)(pAL22)	This study
AKY204	AKY148 *cdc55*::*NAT1*	This study
AKY205	AKY150 *cdc55*::*NAT1*	This study
Y2092	*MAT*α *ade2 his3 leu2 trp1 ura3 GAL*	This study
Y2762	Y2092 *pph21*::*LEU2 pph22*::*HIS3 GAL*	This study
AKY101	Y2092 *MSN2*::pRS306-*MSN2-myc*$_{12}$	This study
AKY103	Y2762 *MSN2*::pRS306-*MSN2-myc*$_{12}$	This study
BY4742	*MAT*α *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ	Research Genetics
AKY198	BY4742 p*ADH-MSN2-GFP*	This study
AKY163	Y2092 *rts1*::*URA3* p*ADH-MSN2-GFP*	This study
AKY185	Y2092 *cdc55*::*LEU2 MSN2*::pRS306-*MSN2-myc*$_{12}$	This study
AKY191	BY4742 *tpd3*::*KanMX* p*ADH-MSN2-GFP*	This study
AKY194	AKY79 *tpd3*::*KanMX*	This study
AKY195	AKY84 *tpd3*::*KanMX*	This study

RESULTS

Msn2p localization and 14-3-3 protein function. A previous report postulated that yeast 14-3-3 proteins anchor Msn2p and Msn4p in the cytoplasm (2); however, the cytoplasmic localization of Msn2p was unaltered in strains (*bmh1*Δ *bmh2*Δ) lacking all 14-3-3 function (16, 29). The Σ strains used in those studies contain an uncharacterized suppressor of the *bmh1*Δ *bmh2*Δ growth defect (31). Because suppression of the *bmh1*Δ *bmh2*Δ growth defect might influence Msn2p localization, we examined Msn2p disposition after conditional loss of 14-3-3 protein function. A functional Msn2-Myc fusion protein remained in the cytoplasm of *bmh1-11*(Ts) *bmh2* cells even after growth was completely arrested (2 h) by a shift to the nonpermissive temperature (Fig. 1A). In contrast, the Msn2-Myc fusion protein was concentrated in the nuclei of *bmh1-11*(Ts) *bmh2* cells after 5 min of osmotic shock (Fig. 1A), indicating that the Msn2-Myc fusion was sensitive to normal physiological stimuli in that background. Thus, the rapamycin-sensitive interaction between Msn2p and Bmh2p (2) may be a simple reflection of the separation of two abundant cytoplasmic proteins by translocation of one protein to a separate compartment.

Although Bmh1p and Bmh2p did not affect Msn2p localization, both proteins have been implicated as positive activators of PKA pathway function (13, 31). However, neither *BMH1* nor *BMH2* suppressed the growth defect of several PKA tem-

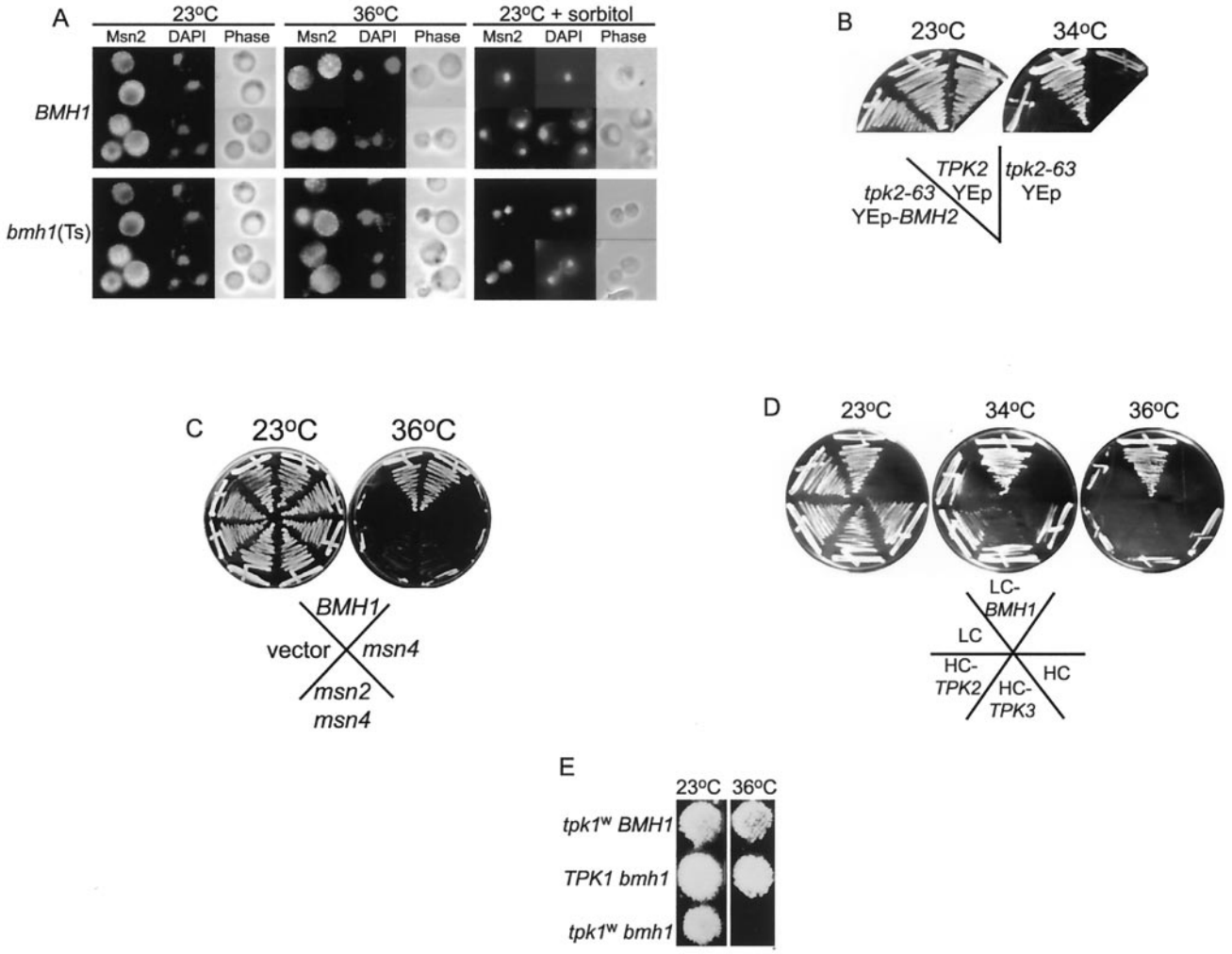

FIG. 1. 14-3-3 protein function and the PKA pathway. A. 14-3-3 protein depletion and Msn2p localization. Wild-type *BMH1* (AKY32) and conditional *bmh1*(Ts) (AKY33) strains containing a functional Msn2-myc$_{12}$ fusion were shifted from 23 to 36°C for 30, 60, and 120 min (60 min is shown) or from 23 to 23°C plus 1 M sorbitol for 5 min, and anti-myc antibody was used to visualize Msn2p by indirect immunofluorescence. Cells were examined by epifluorescence and phase-contrast microscopy. B. 14-3-3 proteins and PKA-dependent growth. The indicated strains were incubated at 23 and 34°C for 3 days. Strains AKY54 (*tpk2-63* YEp-*BMH1*), AKY31 (*TPK2* YEp), and AKY53(*tpk2-63* YEp) are shown. C. PKA pathway and *bmh1-11*(Ts) *bmh2* growth. Isogenic derivatives of conditional *bmh1-11*(Ts) *bmh2*::*URA3* strain SGY459 were incubated at 23 or 36°C for 3 days. Strains AKY1 (vector), AKY2 (*BMH1*), AKY24 (*msn4*Δ), and AKY25 (*msn2*Δ *msn4*Δ) are shown. D. PKA pathway and *bmh1-11*(Ts) *bmh2* growth. Isogenic derivatives of conditional *bmh1-11*(Ts) *bmh2*::*KanMx* strain AKY5 were streaked onto agar and incubated at 23, 34, or 36°C for 3 days. Strains AKY9 (LC-*BMH1*), AKY10 (LC), AKY45 (HC-*TPK2*), AKY46 (HC-*TPK3*), and AKY44 (HC) are shown, where LC and HC refer to low-copy and high-copy plasmids, respectively. E. Synthetic growth defect conferred by PKA and *bmh1* mutations. Strains containing the indicated genotypes were grown at 23°C and and incubated at 23 and 34°C for 3 days. Strains AHY148 (*tpk1*^{w} *BMH1*), AHY240a (*TPK1 bmh1*), and AHY240b (*tpk1*^{w} *bmh1*) are shown.

perature-sensitive mutants [*tpk-63*(Ts) and *tpk2-70*(Ts)] (Fig. 1B and data not shown). Moreover, the *bmh1-11*(Ts) *bmh2* strain accumulated wild-type levels of glycogen at semipermissive temperatures (data not shown). These results suggest that 14-3-3 proteins do not act on Msn2p function or function downstream of PKA. A functional interaction between Bmh1p/Bmh2p and the PKA pathway was also suggested by the observation that elevated PKA activity could suppress the growth defect of cells depleted of 14-3-3 protein (13); however, those studies used a strain in which the only source of 14-3-3 protein was provided by *BMH2* under the control of the glucose-repressed *GAL1* promoter. Because PKA is known to affect carbon metabolism (4, 10), we examined whether the *bmh1-11*(Ts) *bmh2* growth defect could be suppressed by deletion or overexpression of PKA pathway genes *MSN2*, *MSN4*, *YAK1*, *TPK1*, *TPK2*, and *TPK3*. Interestingly, none of these regimens could alleviate the *bmh1-11*(Ts) *bmh2* growth defect (Fig. 1C and D; also data not shown). Indeed, the only genetic interaction observed was a conditional, synthetic growth defect exhibited by a strain containing a deletion of the major 14-3-3 gene, *BMH1* (13), as well as a compromised (wimp) allele of the single, available PKA catalytic gene *TPK1* (Fig. 1E).

Rapamycin stimulates Msn2p translocation by inhibiting Tap42p. Several reports have indicated that rapamycin treatment, like stress, causes Msn2p to accumulate in the nucleus (2, 16); however, rapamycin treatment alone did not elicit

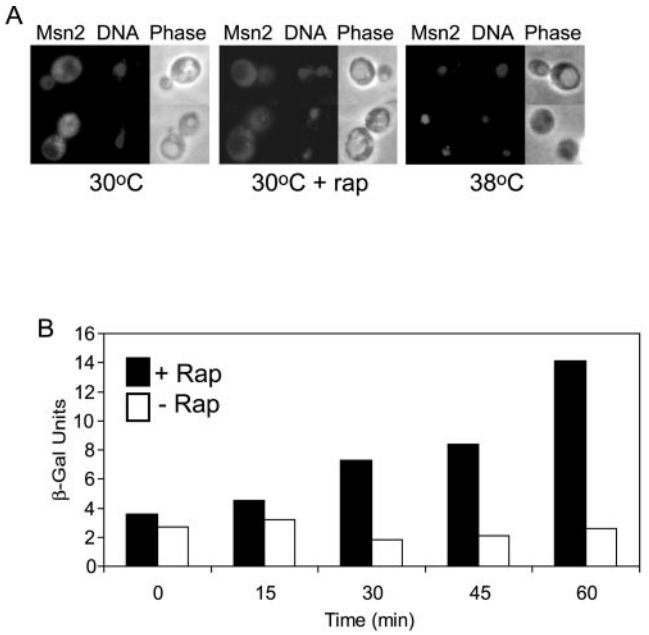

FIG. 2. Rapamycin and Msn2p regulation. A. Rapamycin and Msn2 localization. Wild-type strain AKY34 containing the full-length Msn2-myc$_{12}$ fusion was grown at 30°C to mid-log phase and transferred to 30°C medium, 30°C medium containing 200 ng of rapamycin (rap) per ml, or 38°C medium. Cells were monitored by epifluorescence and phase-contrast light microscopy after 0, 5, 10, 15, 30, and 60 min (pictures shown are from 30 min). B. Rapamycin and *PUT1* expression. The *put1-lacZ* fusion strain AKY35 was transferred to 30°C medium containing 200 ng of rapamycin (Rap) per ml for the indicated times, and β-galactosidase (β-Gal) activity was measured.

nuclear accumulation of functional, full-length Msn2-Myc (9) or Msn2-GFP (Fig. 2A) fusions in several of our lab strains. Nevertheless, although Msn2p-GFP remained in the cytoplasm of cells that were exposed to either rapamycin or mild heat stress (36°C), it accumulated in the nuclei of cells that were transferred to 36°C in medium containing rapamycin (9; also see below). Further, Msn2-GFP rapidly accumulated in the nuclei of cells exposed to heat (38°C [Fig. 2A]) or osmotic shock (see below). This discrepancy with previous results was not due to rapamycin resistance, because cells stopped dividing (data not shown) and rapamycin treatment induced expression of a *put1-lacZ* gene fusion (Fig. 2B) to levels observed previously (17, 34) within 60 min of rapamycin addition.

If rapamycin exerts its effects on Msn2p by inactivating Tap42p, Msn2p should accumulate in the nuclei of conditional *tap42-106*(Ts) mutants at the nonpermissive temperature. We have shown this to be true for the Msn2-Myc fusion protein (9) and confirm that observation with a full-length Msn2-GFP fusion (Fig. 3A). Yeast Tip41 protein interacts with Tap42p and has been proposed to inhibit Tap42p (23). Interestingly, all of our *tap42*(Ts) *tip41*Δ double mutants grow more slowly than the isogenic *tap42*(Ts) *TIP41* parent at both permissive and semipermissive temperatures (Fig. 3B and data not shown). This is distinct from reported results and suggests that Tip41p function might be more complex than previously thought (23). Whatever the explanation for this discrepancy, it is not related to Msn2p function, because the *tap42*(Ts) growth defect was not affected by deletion of *MSN2* and *MSN4* (Fig. 3C). The Msn2-GFP fusion protein remains in the cytoplasm of a *tap42-106*(Ts) *tip41*Δ double mutant at 36°C (Fig. 3A). Deletion of *TIP41* therefore seems to be epistatic to the *tap42*(Ts) mutation. Instead of being an inhibitor of Tap42p, our results suggest that Tip41p plays an active role in transducing the signal between Tap42p and the protein phosphatase complex. Consistent with this hypothesis, the *tip41*Δ mutation also prevented Msn2p from accumulating in the nucleus in response to rapamycin (Fig. 3D).

Tap42p regulation of Msn2p is mediated by PP2A. In contrast to its effects on Msn2p translocation in response to rapamycin and Tap42p depletion, the *tip41*Δ mutation did not affect either conditional growth or Msn2p translocation of the conditional PKA *tpk2-63*(Ts) strain (data not shown). This result suggests that Tap42p affects Msn2p by a PKA-independent mechanism. This conclusion was supported by the observation that a C-terminal Msn2-GFP fusion, which is regulated by PKA but not rapamycin or stress (16), remained in the cytoplasm of *tap42*(Ts) cells even after 60 min at the nonpermissive temperature (data not shown).

If Tap42p inhibits PP2A phosphatase catalytic activity, inactivation of the PP2A catalytic subunits, encoded by redundant genes *PPH21* and *PPH22*, should block Msn2p translocation in rapamycin-treated cells. Interestingly, full-length Msn2-GFP remained in the cytoplasm of the *pph21*Δ *pph22*Δ double mutant when cells were transferred to 36°C in the presence of rapamycin, whereas the same treatment caused Msn2-GFP to accumulate in the nuclei of isogenic single mutants, as well as the wild-type parent (Fig. 4A). The *pph21 pph22*Δ mutant also failed to concentrate Msn2-GFP in the nucleus in response to 1 M sorbitol (Fig. 4B) and heat (38°C [data not shown]), suggesting that Pph21/Pph22 protein phosphatase activity is required for both rapamycin- and stress-stimulated Msn2p translocation. This translocation defect was not due to a general perturbation of Msn2p localization, because Msn2-GFP accumulated in the nucleus of the *pph21*Δ *pph22*Δ strain upon glucose depletion (Fig. 4B). Finally, although Tap42p also regulates the PP2A-like phosphatase catalytic subunit Sit4p (8), the Msn2-GFP fusion protein accumulated in the nuclei of rapamycin-treated *sit4*(Ts) and *sit4*Δ *ssd1*^{v} strains that were shifted to 36°C (data not shown), in agreement with a previous claim that Sit4p does not play a role in Msn2p localization (2).

Msn2p accumulation requires the PP2A regulatory subunit Cdc55p. In addition to the catalytic subunits, yeast PP2A phosphatase is comprised of a regulatory subunit, which can be either Rts1p or Cdc55p, and a scaffolding protein, Tpd3p. Strains lacking either of the regulatory subunits exhibit distinct phenotypes, whereas a *tpd3*Δ mutant displays phenotypes that appear to be a composite of the two regulatory mutants (44). Mutants lacking individual subunits were treated with rapamycin or stress to determine their contribution to Msn2p localization. Both *tpd3*Δ (Fig. 5A) and *cdc55*Δ (Fig. 5B) cells retained Msn2p in the cytoplasm upon treatment with rapamycin (rapamycin and 36°C) or 1 M sorbitol, whereas Msn2p quickly translocated to the nuclei of *rts1*Δ and wild-type cells under the same regimens (Fig. 5A and B). Thus, scaffolding protein Tpd3p and regulatory protein Cdc55p are required for rapamycin- and stress-induced Msn2p translocation.

Expression of Msn2-dependent genes is elevated upon stress

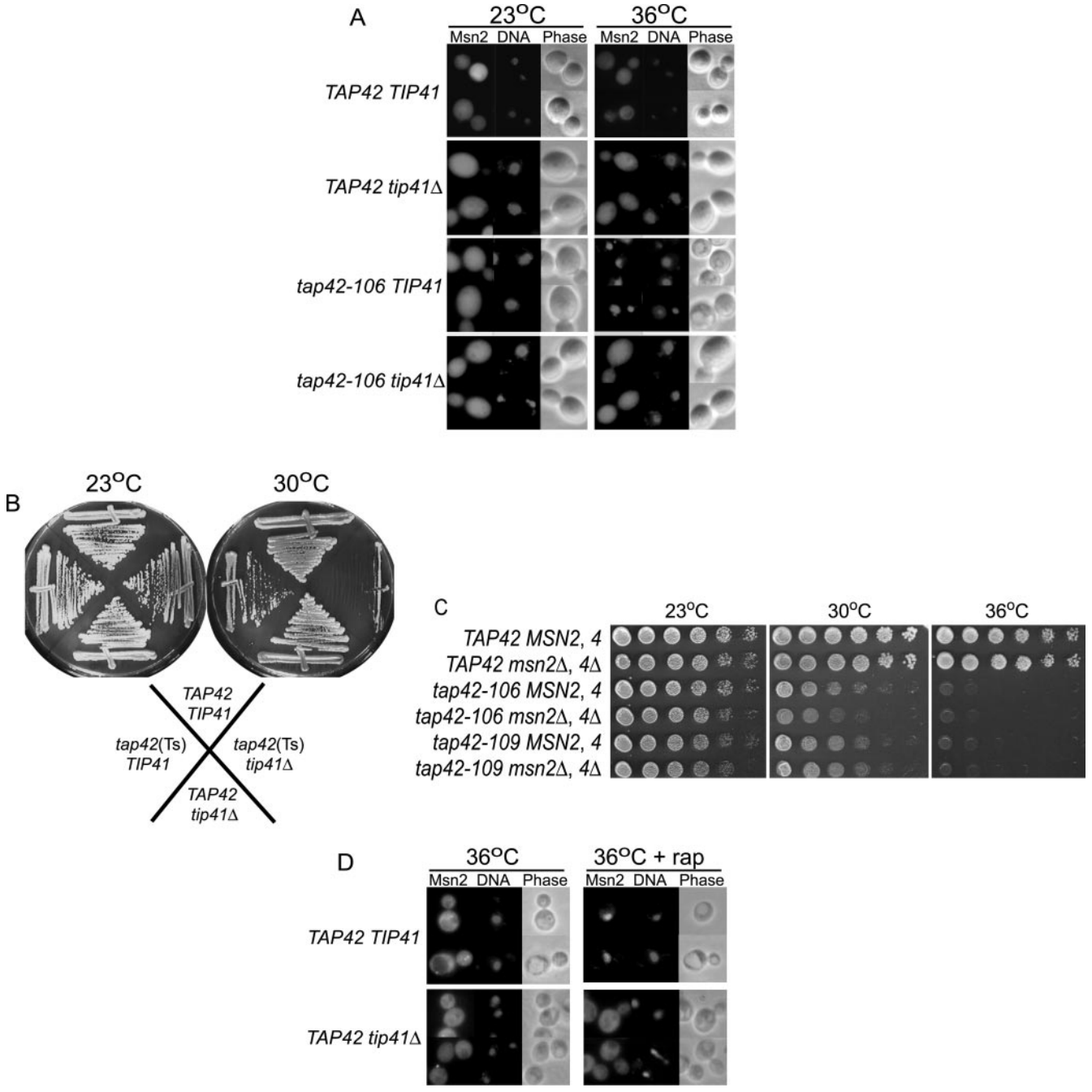

FIG. 3. The Tap42p-interacting protein Tip41p influences Msn2p localization. A. *tip41*Δ prevents Msn2p nuclear accumulation in a *tap42*(Ts) mutant. Strains containing full-length Msn2-GFP fusion were grown at 23°C to mid-log phase and transferred to 36°C medium. After 30 min, cells were viewed by epifluorescence and phase-contrast light microscopy. In panels A and B, strains AKY79 (*TAP42 TIP41*), AKY84 [*tap42-106*(Ts) *TIP41*], AKY142 (*TAP42 tip41*Δ), and AKY144 [*tap42-106*(Ts) *tip41*Δ] are shown. B. *tip41*Δ and *tap42*(Ts) growth. Strains from panel A were incubated for 3 days at 23 and 30°C. C. Msn2 and *tap42*(Ts) growth. Strains were incubated for 3 days at 23, 30, and 36°C. Strains Y3033 (*TAP42 MSN2,4*), Y3036 (*TAP42 msn2*Δ,*4*Δ), Y3034 (*tap42-106 MSN2,4*), Y3037 (*tap42-106 msn2*Δ,*4*Δ), Y3034 (*tap42-109 MSN2,4*), Y3038 (*tap42-109 msn2*Δ,*4*Δ) are shown. D. *tip41*Δ prevents Msn2p nuclear accumulation in response to rapamycin. Strains were grown at to mid-log phase at 23°C and transferred to 36°C medium either lacking or containing rapamycin (200 ng/ml). Strains AKY36 (*TAP42 TIP41*) and AKY75 (*TAP42 tip41*Δ) are shown.

or nutrient starvation as a result of the accumulation of Msn2p in the nucleus. To determine whether PP2A-deficient cells are refractory to stress-induced stimulation of Msn2-dependent genes, we monitored expression levels of a *CTT1-lacZ* reporter fusion. Whereas Tap42p inactivation normally resulted in five-fold induction of *CTT1-lacZ* expression, this increase was totally dependent upon Cdc55p function (Fig. 5C). Similar results were observed when wild-type and *cdc55*Δ cells were

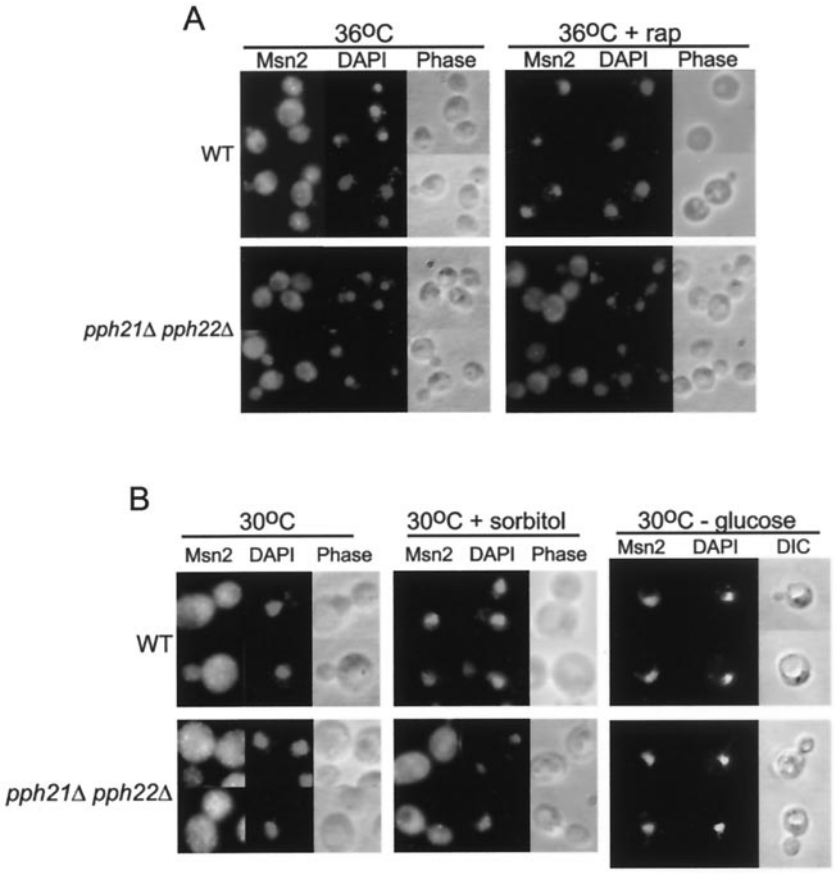

FIG. 4. PP2A activity and Msn2p translocation in response to rapamycin and stress. A. PP2A catalytic activity is required for Msn2p translocation in response to rapamycin. Strains containing the Msn2-myc$_{12}$ fusion were grown to mid-log phase at 23°C and transferred to 36°C medium lacking or containing 200 ng of rapamycin per ml. After 30 min, cells were viewed by epifluorescence and phase-contrast light microscopy. Strains AKY101 (wild type [WT]) and AKY103 (*pph21*Δ *pph22*Δ) are shown. B. PP2A catalytic activity is required for Msn2p translocation in response to stress. Strains shown in panel A were grown to mid-log phase in medium containing 2% glucose and then transferred to the same medium, the same medium containing 1 M sorbitol, or the same medium lacking glucose. After 30 min (10 min for glucose depletion), cells were visualized by epifluorescence, phase-contrast light, and differential-contrast (DIC) microscopy.

treated with osmotic shock (Fig. 5D), implying that PP2A activity is required for stress-activated localization and function of Msn2p.

Nitrogen starvation regulates Msn2p localization by a PP2A-dependent mechanism. In contrast to glucose availability, which is generally thought to modulate Msn2p localization through its influence on the cAMP-dependent protein kinase signaling pathway (4, 16, 29), there is no generally accepted model that accounts for the effects of nitrogen starvation on Msn2p localization. One model posits that nitrogen levels are sensed and transmitted to the PKA catalytic subunit by a poorly defined, cAMP-independent mechanism referred to as the FGM (fermentable-growth-medium-induced) pathway. The FGM model was proposed to explain how nitrogen starvation could affect Msn2-regulated processes in the absence of an effect on cAMP levels; however, there is no direct evidence to suggest that either PKA activity or Msn2p phosphorylation corresponds to nitrogen availability.

An alternative model is that the TOR kinase pathway, which is known to respond to nitrogen levels, transmits the nitrogen starvation signal to Msn2p (2, 16). Despite the attraction of the model, Msn2p localization has not been examined in either wild-type or mutant cells starved of nitrogen. Interestingly, full-length Msn2-GFP accumulated in the nuclei of wild-type cells within 30 min of nitrogen starvation (Fig. 6), whereas it took at least 2 h to exhibit nuclear accumulation of the carboxyl-terminal Msn2-GFP fusion in a minor fraction of the cells (data not shown). The full-length Msn2-GFP fusion also accumulated in the nuclei of *tpk1*^{w2} *bcy1* cells after 30 min of nitrogen starvation (Fig. 6), whereas *tpd3*Δ cells displayed cytoplasmic accumulation at least 2 h after nitrogen starvation (Fig. 6). In contrast, the full-length Msn2-GFP fusion quickly accumulated in the nuclei of glucose-depleted *tpd3*Δ cells (Fig. 6), indicating that the strain's lack of response to nitrogen was not a general defect in nutrient sensing or response. Thus, nitrogen limitation affects the amino-terminal, stress-sensitive domain of Msn2p by a mechanism that requires PP2A phosphatase activity and is independent of PKA regulatory protein Bcy1p.

DISCUSSION

Accumulation of Msn2p in the nucleus and subsequent activation of stress response genes results from a balance between nuclear import and nuclear export. Görner and col-

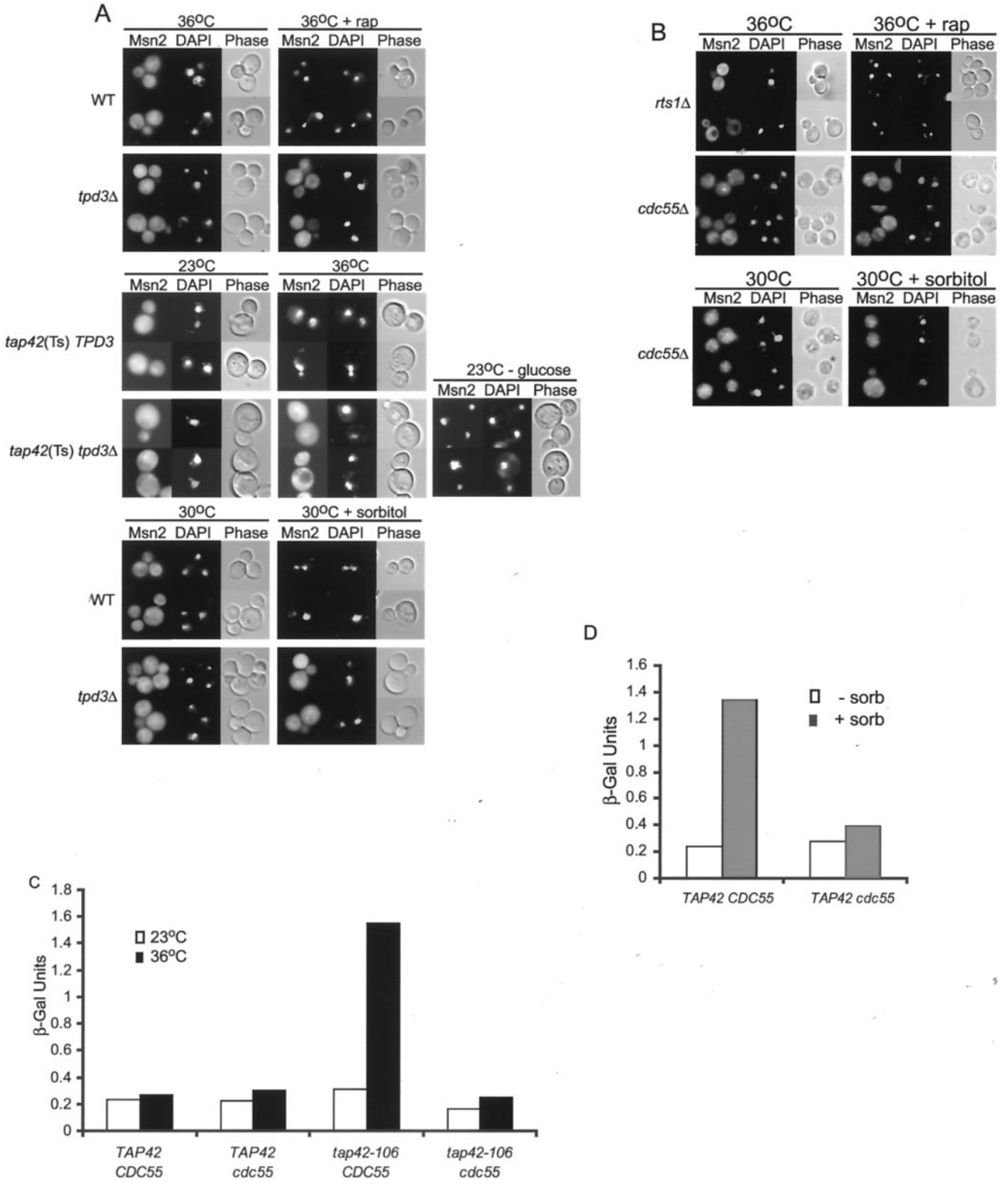

FIG. 5. PP2A regulatory subunits and Msn2p translocation. A. PP2A scaffolding subunit Tpd3p is required for Msn2p translocation in response to rapamycin (rap), Tap42p depletion, and stress. Strains containing the full-length Msn2-GFP fusion protein were treated as described in the legend to Fig. 4 (23°C - glucose, 23°C medium lacking glucose) and visualized by epifluorescence and phase-contrast light microscopy. Strains AKY198 (wild type [WT]), AKY191 (*tpd3*Δ), AKY194 [*tap42*(Ts) *TPD3*], and AKY195 [*tap42*(Ts) *tpd3*Δ] are shown. B. PP2A regulatory subunit Cdc55p is required for Msn2p translocation in response to rapamycin and stress. Strains AKY163 (*rts1*Δ) and AKY185 (*cdc55*Δ) containing full-length Msn2-GFP and Msn2-myc$_{12}$ fusion proteins, respectively, were treated as described in the legend to Fig. 4 and visualized by epifluorescence and phase-contrast light microscopy. C. PP2A function and Msn2-dependent gene expression in response to Tap42p inactivation.

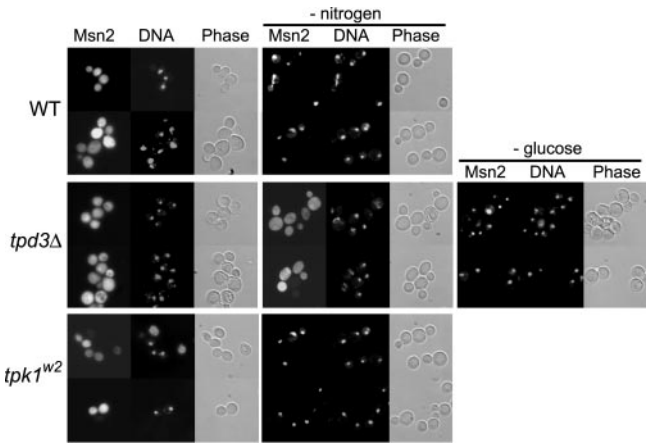

FIG. 6. PP2A activity is essential for Msn2p nuclear accumulation in response to nitrogen starvation. Strains containing the full-length Msn2-GFP fusion protein were grown in selective minimal medium at 30°C before they were transferred to the same medium or the same medium lacking (−) nitrogen or glucose for 30 min. Strains AKY198 (wild type [WT]), AKY191 (*tpd3*Δ), and RTF1.5-2 (*tpk1*^{w2}) are shown. Cells were examined by epifluorescence or phase-contrast microscopy.

leagues recently identified a carboxyl-terminal domain that is responsible for glucose-responsive nuclear import, as well as an amino-terminal region that is thought to promote nuclear export in response to glucose and stress (16). Our results help clarify the signaling pathway that impinges on the export process.

Our results indicate that the Tor kinase pathway regulates Msn2p localization through its effects on PP2A protein phosphatase. Because Tor kinase function does not affect the carboxyl-terminal NLS (16; data not shown), these results are most consistent with a model in which Tor kinase and PP2A regulate nuclear export of Msn2p. That is, the PP2A catalytic subunit encoded by *PPH21*/*PPH22* in a complex with the regulatory subunit Cdc55p and the scaffold protein Tpd3p inhibits export of Msn2p from the nucleus. During mitotic growth, Tor-activated Tap42p suppresses the activity of this phosphatase complex and stimulates Msn2p export from the nucleus. When Tor kinase is inactivated by the addition of rapamycin, PP2A activity is derepressed, and Msn2p export is inhibited. Thus, Tor inactivation causes Msn2p to accumulate in the nucleus through the trapping of a normal low-level flux of Msn2p into the nucleus. There is at least one other example of a phosphatase modulating the localization of a yeast transcription factor. In response to mating pheromone, cytosolic calcium levels increase and activate the calcium-calmodulin-dependent phosphatase calcineurin. Activated calcineurin then dephosphorylates the transcription factor Crz1p, which accumulates in the nucleus and stimulates expression of genes needed for cell wall remodeling and survival (3).

Tor inactivation, stress, and nitrogen starvation result in accumulation of Msn2p in the nucleus and prolonged activation of stress response genes (2, 9, 16, 29) (Fig. 6). The parsimonious conclusion is that all of these conditions act on the same mechanism of nuclear export. In contrast to glucose depletion, which regulates both the amino- and carboxyl-terminal domains of Msn2p, Tor inactivation, stress, and nitrogen depletion affect only the amino-terminal domain (9, 16). This correlation is further strengthened by our observation that PP2A activity is essential to Msn2p regulation by the latter stimuli, whereas Msn2p responds to glucose depletion independent of PP2A function (Fig. 4 and 5). This specificity also argues against the possibility that PP2A phosphatase activity plays an indirect role in Msn2p localization. Thus, we propose that cells sense and respond to stress (and Tor inactivation) by stimulating PP2A-dependent dephosphorylation and inhibiting export of Msn2p. Finally, although glucose depletion could also affect nuclear export of Msn2p by a PP2A-independent mechanism, a simpler model would be one in which glucose depletion regulates nuclear import of the amino-terminal region in much the same way that it regulates the carboxyl-terminal NLS (16). In this latter scenario, the amino-terminal domain would contain a glucose-sensitive NLS in addition to the stress-sensitive NES.

Even if Tor kinase does not transmit the stress signal, stress conditions could still be transduced to PP2A phosphatase by a separate signaling pathway. Little is known about the regulation or function of the individual PP2A subunits; however, it is interesting that Cdc55p, but not Rts1p, is required for Msn2p translocation (Fig. 5). These results imply that Cdc55p performs a PP2A targeting function that is separate from that of Rts1p, a conclusion that is consistent with the notion that these two proteins influence independent cellular processes (14, 19, 37). The independence of these regulatory subunits underscores the possibility that they are regulated by distinct physiological stimuli and regulatory mechanisms. Thus, stress and rapamycin (Tor kinase) could impinge upon PP2A phosphatase activity by convergent mechanisms, rather than by a linear pathway in which stress inhibits Tor kinase activity.

Bmh1p/Bmh2p and the PKA pathway. Beck and Hall (2) presented a model in which yeast 14-3-3 protein (Bmh1p/Bmh2p) acted as a cytoplasmic anchor for the transcription factor Msn2p. Evidence supporting this model included genetic interactions between 14-3-3 genes *BMH1*/*BMH2* and the PKA pathway (13, 31), a rapamycin-sensitive interaction between Bmh2p and Msn2p (2), and a previously documented role for mammalian 14-3-3 proteins in cytoplasmic sequestration of signaling proteins (30). However, this model was incon-

β-Galactosidase (β-Gal) activities of the indicated *CTT1-lacZ* strains were measured during exponential growth at 23°C and 30 and 45 min (shown) after the shift to 36°C. Values shown are the mean of four measurements from two separate experiments. All standard deviations were less than 15%, with the exception of the 23°C measurement of the *tap42-106 cdc55* strain, which was less than 35%. Strains AKY148 (*TAP42 CDC55*), AKY204 (*TAP42 cdc55*), AKY150 (*tap42-106 CDC55*), and AKY205 (*tap42-106 cdc55*) are shown. D. PP2A function and Msn2-dependent gene expression in response to osmotic shock. β-Galactosidase activities of the indicated *CTT1-lacZ* strains were measured during exponential growth in minimal medium and 30 min after the shift to minimal medium containing 1 M sorbitol (sorb). Values shown are the mean of four measurements from two separate experiments. All standard deviations were less than 15%. Strains AKY148 (*TAP42 CDC55*) and AKY205 (*TAP42 cdc55*) are shown.

sistent with several recent observations that Msn2p remained in the cytoplasm of a strain (Σ *bmh1*Δ *bmh2*Δ) lacking all 14-3-3 proteins (16, 29). Yeast 14-3-3 function is normally essential for viability (13, 31, 43), implying that the Σ *bmh1*Δ *bmh2*Δ strain used in the Msn2p localization studies (16, 29) contained a suppressor of the growth defect. Although the *bmh1*Δ *bmh2*Δ growth suppressor might have influenced Msn2p localization, our results (Fig. 1A) are consistent with the conclusion that 14-3-3 protein does not act as a cytoplasmic anchor of Msn2p. Yeast 14-3-3 proteins are relatively abundant and interact with a large number of proteins by both biochemical and two-hybrid techniques (11, 21, 22, 42). Thus, their association with Msn2p might simply reflect a nonspecific interaction, and the rapamycin sensitivity of this interaction could, in turn, be rationalized by a change in cellular distribution of one (Msn2p) of these factors.

If 14-3-3 proteins do not anchor Msn2p in the cytoplasm, what is their role in PKA-dependent processes? Genetic studies showed that 14-3-3 proteins stimulated PKA-dependent growth and pseudohyphal formation (13, 31), and several observations were consistent with the notion that they impinged upon the pathway between the upstream components Cdc25p and Ras2p. For example, while Bmh1/Bmh2 overexpression suppressed the growth defect of a *cdc25*(Ts) mutant, lesions in the downstream Ras2p protein were unaffected by overproduction of either 14-3-3 protein (13). Moreover, PKA-regulated processes remained responsive to the activated $Ras2^{G2V}$ allele in a *bmh1*Δ *bmh2*Δ strain, implying that 14-3-3 proteins do not act downstream of Ras2p protein function (31). Although our observation that *BMH1*/*BMH2* overexpression cannot suppress the *tpk2*(Ts) growth defect is consistent with this interpretation, it is hard to reconcile such a model with our observation that Bmh1/Bmh2 inactivation did not affect Msn2p localization (Fig. 1). Because Ras and PKA function are required to maintain Msn2p in the cytoplasm (15), this result would seem to rule out a role for yeast 14-3-3 proteins in regulating Ras/PKA function. Taken together, these results are most consistent with a model in which 14-3-3 proteins impinge upon several PKA-regulated processes by an unknown, or at least PKA-independent, pathway. Support for this conclusion also comes from our observation that PKA activation (by overexpression of each of the three *TPK* genes or inactivation of *MSN2*/*MSN4* or *YAK1*) failed to alleviate the *bmh1*(Ts) *bmh2* growth defect (Fig. 1).

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