

A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation

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In yeast, glucose depletion elicits a quick response in the transcription of stress-related genes. The main transcriptional activator that orchestrates this response is Msn2, whose nuclear localization and DNA binding are negatively controlled by the cAMP-dependent protein kinase (PKA). Msn2 activation by sudden glucose depletion correlates with a fast but transient decrease in phosphorylation of several sites in its nuclear localization signal (NLS). Here we show that protein phosphatase 1 (PP1) is the direct antagonist of PKA-dependent phosphorylation at the Msn2 nuclear import domain and therefore a potential mediator of glucose starvation signals that target this transcription factor. Apart from PKA, the protein kinase Snf1 can also directly modify one of the Msn2 phosphorylation sites (S582) and thereby repress Msn2 function. Consequently, in *snf1* mutants, rephosphorylation of the NLS happens to be much slower during prolonged starvation. Thus, a second, Reg1-dependent form of PP1 indirectly influences Msn2 functionality by modulating Snf1 kinase activation and repression. Different activities of PP1 are therefore involved in shaping induction and adaptation of the transcriptional stress response during acute glucose starvation.

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Introduction

Within their natural habitat, yeast cells have to make many metabolic adjustments in response to changes in extracellular nutrient conditions. Such alterations are generally associated with broad effects on gene expression. In a detrimental growth environment genes involved in glucose metabolism are downregulated, whereas genes such as *HSPs* and *CTT1*, associated with stress protection mechanisms, become upre-

gulated (Rep *et al*, 1999; Gasch *et al*, 2000). Still, yeasts must always be ready to quickly return to optimal metabolic means when the milieu becomes favorable, emphasizing the importance of adaptive mechanisms (Kaniak *et al*, 2004). Under standard culture conditions, when supplied with sufficient nutrients, including a fermentable carbon source such as glucose, yeast cells first grow exponentially until glucose levels become depleted. Ultimately, cells will reach stationary phase and enter into a quiescent state. All these relatively slow growth transitions are associated with major changes in transcription. A sudden lack of glucose in exponentially growing cells will also cause a major shift in gene expression. Its pattern mimics to a considerable extent the pattern observed under environmental stress situations (Gasch *et al*, 2000), suggesting that glucose deprivation and stress might target a common regulatory node.

Among the protein kinases implicated as key elements in the nutritional transitions mentioned above, PKA plays a central role in growth-related phenomena during fermentation. By targeting certain transcription factors, PKA also controls the induction of growth-related genes and the repression of stress-related genes. For instance, a defect in the PKA inhibitory subunit Bcy1 leads to high constitutive kinase activity and causes sensitivity to starvation and stress, whereas cells with weak cAMP production and therefore low PKA activity (e.g. *ras2* mutants) display hyperaccumulation of glycogen and higher stress resistance (Rolland *et al*, 2002).

In contrast, Snf1, the ortholog of the mammalian AMP-activated protein kinase in *Saccharomyces cerevisiae* (*S.c.*), has no well-defined function for glucose-growing cells, although it assumes a central role during glucose derepression (Jiang and Carlson, 1997). High glucose levels normally inhibit the expression of multiple genes including those involved in the utilization of alternative carbon sources, gluconeogenesis, and respiration. Glucose repression is established and maintained by the transcription factor Mig1 and its corepressors (Carlson, 1999). While Mig1 is inactivated by the Snf1 kinase complex, Snf1 is in turn activated upon glucose depletion (Wilson *et al*, 1996). Additionally, the nutrient-regulated protein phosphatase 1 (PP1) complex plays an important role by negatively regulating Snf1 upon readdition of glucose (Sanz *et al*, 2000). Besides its catalytic subunit Glc7, the PP1-targeting subunit Reg1 plays a key role in this process, since *reg1* cells are unable to establish glucose repression while exhibiting constitutive levels of active Snf1. The protein phosphatase acting on Mig1 upon addition of glucose has not been identified so far. Indirect evidence suggests PP1, as Glc7–Reg1 complex, to be a candidate because Mig1 is constitutively phosphorylated and cytosolic both in *reg1* and *glc7* cells (Treitel *et al*, 1998; McCartney and Schmidt, 2001). Alternatively, constitutive Snf1 activity could explain the constant phosphorylation of Mig1 in *reg1* cells. Glucose signals, therefore, seem to have opposite effects towards activation of Snf1 and PKA. Indeed, many pheno-

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types associated with the loss of Snf1 function are similar to those allied with PKA hyperactivity (Hubbard *et al*, 1992).

During exponential growth and throughout the diauxic growth transition, the transcriptional adjustment of stress-protecting genes is coordinated by the two closely related transcription factors Msn2 and Msn4. Indeed, variations in Msn2 localization and activity have been used as a sensitive indicator of nutrient fluctuations and environmental stress conditions (Schmitt and McEntee, 1996). According to several DNA microarray analyses, the Msn2-regulon may consist of more than 150 genes (Gasch *et al*, 2000; Causton *et al*, 2001). High PKA activity normally holds this STress Responsive Element (STRE)-driven gene expression down and appears to set a threshold response to environmental stress signals (Marchler *et al*, 1993; Görner *et al*, 1998). Immune fluorescence studies with Msn2-GFP chimeras showed that most of Msn2 resides in the cytosol when PKA activity is high. However, either stress or acute glucose depletion induces rapid nuclear accumulation of Msn2 (Görner *et al*, 1998), an effect found to be reversible by simple removal of the stress factor or by increasing PKA activity. A nuclear export sequence (NES) was identified in the Msn2 protein, and its functionality was demonstrated to be dependent on the nuclear exportin Msn5 (Görner *et al*, 2002). Increased nuclear import can apparently also be achieved by dephosphorylation of sites in the Msn2 nuclear localization signal (NLS). This event occurs upon downregulation of PKA or under acute glucose deprivation. In fact, the identity of the phosphorylation sites has been determined and the direct involvement of PKA is well documented (Görner *et al*, 2002). Nuclear import of Msn2 is dependent on the karyopherin Kap123 that may preferentially interact with the nonphosphorylated form of the Msn2-NLS (W Görner, unpublished data). Finally, there is evidence that STRE binding of nuclear Msn2 is also induced by stress and antagonized by PKA, but the underlying mechanisms are not well defined (Görner *et al*, 1998).

The rapid dephosphorylation of Msn2 under glucose depletion suggests that protein phosphatases must play an important role in the activation of this transcription factor. However, the relatively transient nature of dephosphorylation under prolonged glucose depletion hinted at a more complex regulatory network that could include additional players at the protein kinase and/or the protein phosphatase level. Indeed, previous genetic and cell biological observations have implicated PP1 as well as Snf1 in the regulatory networks around Msn2 function, as high copy numbers of the transcription factor could suppress certain Snf1 defects (Estruch and Carlson, 1993). More recently, Sanz and colleagues reported genetic evidence for the participation of Snf1 in the inhibition of Msn2 nuclear accumulation in response to glucose depletion (Mayordomo *et al*, 2002). However, neither the element responsive to the inactivation by Snf1 was identified, nor was a direct biochemical relationship to the kinase or its phosphatase regulator established. PP1 plays an important role in regulating various processes in eukaryotic cells. In budding yeast, Glc7 was demonstrated to be required for the regulation of, for instance, translation, cell cycle progression, chromosome segregation, glycogen accumulation, and glucose repression (Ceulemans and Bollen, 2004). In order to keep these regulation mechanisms independent, specialized targeting subunits are thought to restrict the function of the phosphatase to certain substrates (Bollen,

2001). For example, Reg1 was primarily identified as a cytosolic targeting factor that specifies inactivation of the Snf1 kinase by PP1 in glucose-growing cells. Glucose depletion has therefore been connected with the inactivation of the Reg1-dependent PP1 complex (Ludin *et al*, 1998; Sanz *et al*, 2000). More recently, the Glc7-Bud14 complex has been positively implicated in the regulation of Msn2-driven gene transcription during the diauxic shift (Lenssen *et al*, 2005); however, the target of this phosphatase complex remains unidentified. Here we focus on clarifying the function of Snf1 and PP1 towards one of the transport domains of Msn2, namely, the Msn2-NLS.

Results

The NLS of Msn2 contains a putative Snf1 kinase motif

The transcription factor Msn2 contains 704 amino acids with a NES, a NLS enclosing at least four phosphorylation sites, a zinc-finger region and several, but not well-defined, activation domains (Görner *et al*, 2002). *In silico* search for potential phosphorylation sites in Msn2 revealed that one PKA modification site overlapped with a Snf1 consensus motif (Dale *et al*, 1995) at amino acid 582 within the NLS. Since the NLS phosphorylation has been shown to correlate with inhibition of Msn2 import function, phosphorylation by Snf1 was also expected to attenuate Msn2 function. To further analyze the system, we raised antibodies specifically directed against phosphoserines of the Msn2 protein, namely, S582 and S620. Using these specific reagents on extracts from wild-type cells, we could demonstrate that both sites of Msn2-NLS, S582 and S620, are phosphorylated during growth on glucose, while rapidly becoming dephosphorylated upon acute glucose depletion. Both sites were rephosphorylated when glucose was added to the cell cultures, or after prolonged starvation (Figure 1A). According to previous data, it was thought that PKA was mainly responsible for the phosphory-

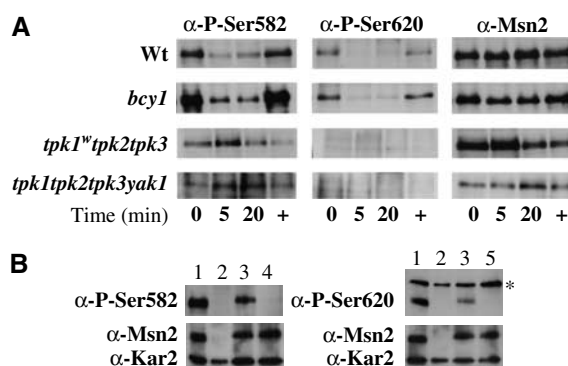


Figure 1 PKA activity is not the sole regulator for the phosphorylation pattern of the Msn2-NLS upon acute glucose depletion. (A) Western analyses were performed with crude protein extracts obtained from wild type (wt), *bcy1*-, *tpk1^wtpk2tpk3*-, or *tpk1tpk2tpk3yak1*-mutant cells before (0) and during acute glucose depletion (5, 20), and 20 min after glucose re-addition (+). Endogenous levels of Msn2 were detected with polyclonal α -Msn2 antibodies. Msn2 phosphorylation was monitored with the specific antibodies α -P-S582 and α -P-S620. (B) Western analyses with protein extracts from wild type (1), *msn2msn4*-mutants (2), and mutants expressing Msn2 (3), Msn2-S582A (4), or Msn2-S620A (5) with α -P-S582, α -P-S620, and α -Msn2 antibodies. Loading was monitored with α -Kar2 antibodies. The α -P-S620 antibodies recognize an additional protein unspecifically at 150 kDa (*).

lation and inactivation of Msn2 (Görner *et al*, 2002). In agreement with this model, we found that in cells devoid of PKA activity (*tpk1^wtpk2tpk3* and *tpk1tpk2tpk3yak1*) Msn2 is still present but not phosphorylated on S620 (Figure 1A). Interestingly, a certain level of phosphorylation was still noticeable on S582 in these cells, suggesting that other protein kinases might also be able to recognize this site *in vivo*.

If PKA is solely responsible for the inactivation of Msn2, then, in a strain lacking the Bcy1 inhibitory subunit of PKA, Msn2 is expected to remain phosphorylated and inactive. Yet, we show that both S582 and S620 are still rapidly dephosphorylated in *bcy1* cells (Figure 1A). Our observations suggest that either a phosphatase must activate the Msn2-NLS or that glucose starvation signals might also target the catalytic subunits of PKA. The loss of Western signal with protein extracts from *msn2msn4* cells or from these cells expressing Msn2 with single alanine substitutions for S582 or S620 indicated that the antibodies used were specific for the phosphorylated forms of Msn2 at the respective sites (Figure 1B).

PP1 is involved in the regulation of Msn2

To identify Msn2-targeted protein phosphatases, we searched for mutant strains unable to import Msn2-GFP into the nucleus upon glucose depletion. An ordered collection of deletion strains served as source for assaying candidate mutants (Supplementary Figure S2). We found that most mutations tested had no noticeable effect on Msn2 localization upon glucose depletion. Many regulatory subunit mutants of PP1, such as *glc8* or *gac1*, had no defect with regard to the regulation of Msn2 nuclear trafficking (data not shown). However, *reg1* cells were severely affected in their response to acute glucose starvation, even though the cells could partially import Msn2-GFP upon osmotic shock (0.5 M NaCl). Interestingly, Reg1 was depicted as an important regulator of glucose signals towards Snf1 (Ludin *et al*, 1998) and was also identified as an effector of Msn2-driven transcription independently of our studies (Mayordomo *et al*, 2002). Overall, our observations were in good agreement with those reported by this lab.

We therefore tested whether an impediment of the dephosphorylation process at PKA-dependent modification sites could explain the cytoplasmic retention of Msn2. Figure 2A shows that in *reg1* cells the Msn2-NLS remains predominantly phosphorylated even upon glucose depletion. Interestingly, this effect was noticeable for both of the sites tested. The role played by Reg1 in the regulation of the Msn2-NLS could be due to an indirect role of PP1 (Glc7-Reg1) through its effect on the Snf1 kinase, but also due to a direct role, possibly shared with other PP1 complexes. We therefore investigated the effects of a mutated version of Glc7, *glc7-T152K*, that is ineffective in binding Reg1 and supposedly affects the role of PP1 in Snf1 repression (Tu and Carlson, 1994, 1995). Once activated by glucose depletion, Snf1 was proposed to be maintained in its active state in this strain. As shown in Figure 2A, Msn2-S582 remained phosphorylated even when glucose was depleted from *glc7-T152K* cultures, whereas S620 was still dephosphorylated under the same conditions. Using a double-mutant strain *glc7-T152Ksnf1*, we could confirm that the effect on S582 was solely due to the activity of Snf1 (Figure 2A) and that the mutated form of the PP1 catalytic subunit did not affect Msn2-NLS dephosphory-

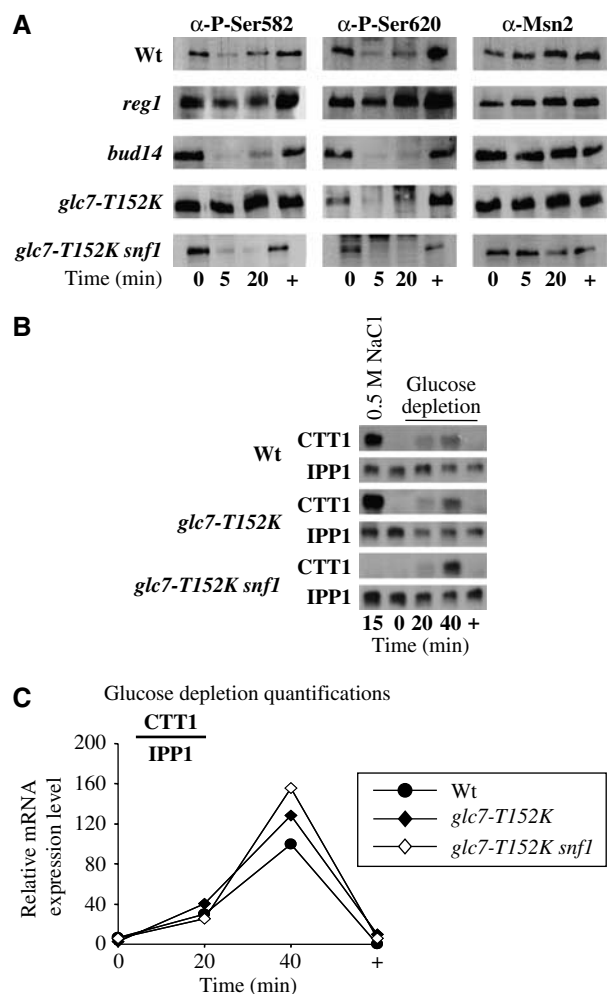


Figure 2 Glc7 and Snf1 participate in the regulation of Msn2. (A) Unregulated Snf1 activity (*glc7-T152K* and *reg1*) leads to aberrant phosphorylation of the Msn2-NLS. Wild type (wt), *reg1*-, *bud14*-, *glc7-T152K*- and *glc7-T152Ksnf1*-mutant cells were grown and treated as described in Figure 1, as were the subsequent Western analyses. (B) Constitutive Snf1 activity does not prevent Msn2 activation upon acute glucose depletion. Wt (—●—), *glc7-T152K* (—◆—), and *glc7-T152Ksnf1* (—◇—)-mutant cells were grown to mid-exponential phase (0) and either treated with 0.5 M NaCl for 15 min (15) or subjected to acute glucose depletion for 20 and 40 min (20, 40), followed by immediate re-addition of glucose; a final sample was taken after 20 min (+). Total RNA was analyzed simultaneously for the expression of the STRE-driven gene *CTT1* and the house-keeping gene *IPP1*. (C) The expression of *CTT1* before, during, and after glucose depletion was normalized for each time point against the corresponding *IPP1* expression level. Quantifications were plotted as a function of the highest expression level in wt cells, which was set to 100.

lation. Moreover, we could show that Bud14 had no role in the dephosphorylation of the Msn2-NLS under the condition tested (Figure 2A).

To test whether the aberrant phosphorylation of Msn2 in *glc7-T152K* mutants had consequences for its physiological function, we followed the expression of *CTT1* upon glucose depletion (Figure 2B and C). Our results show that Msn2 is still active in *glc7-T152K* mutants as compared to wild-type cells. This suggests that the inability of *glc7-T152K* to interact with Reg1 does not hinder Msn2 function. The additional deletion of *SNF1* has no effect on *CTT1* transcriptional induction upon glucose depletion, suggesting that Snf1 is

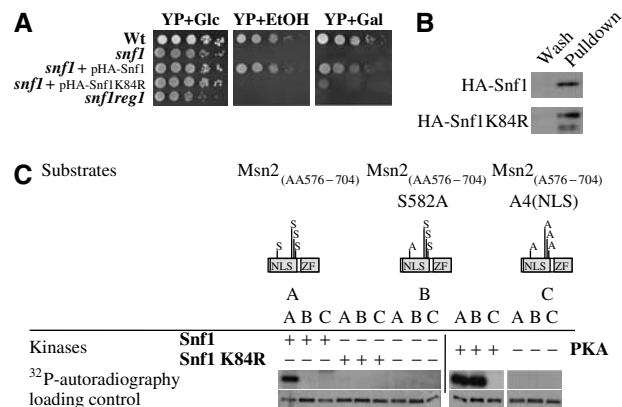


Figure 3 Msn2 is substrate for Snf1 *in vitro*. (A) Snf1 activity is required for growth on nonfermentable carbon sources. Wild type (wt), *snf1*-, *snf1*-expressing Snf1-HA, or Snf1-K84R-HA and *reg1snf1*-mutant cells were grown to OD_{600 nm} = 1.0. Then, 10-fold serial dilutions were spotted onto a rich medium (YP) with either a fermentable (glucose—Glc) or nonfermentable carbon source (ethanol—EtOH or galactose—Gal). Plates were incubated at 30°C for 3 days. (B) Snf1 and Snf1-K84R were purified from crude protein extracts obtained from exponentially grown *snf1*-mutant cells expressing either Snf1-HA or Snf1-K84R-HA and analyzed by Western blot of equal aliquots of the last washes and the eluates, with monoclonal α-HA antibodies. (C) Equal amounts of the substrates GST-Msn2_(AA576-704) (A), GST-Msn2_(AA576-704)S582A (B) and GST-Msn2_(AA576-704)A4(NLS) (C) were incubated with [γ -³²P]ATP and Snf1, Snf1-K84R, or PKA. Control assays (lanes 7–9 and 13–15) lacked the appropriate purified kinase. Samples were separated by SDS-PAGE and analyzed by autoradiography and silver staining.

specifically required for inactivation of Msn2 after prolonged glucose depletion. It is interesting to note that *glc7-T152Ksnf1* double mutants are unable to respond to osmotic stress, supporting the idea that Snf1 plays a decisive role in stress-induced signal transduction (Sanz, 2003).

Snf1 directly phosphorylates serine 582 of Msn2-NLS

Considering that S582 was embedded in an Snf1 consensus motif, we attempted to show that Msn2 is a substrate of this kinase. We transformed *snf1* cells with plasmids expressing either HA-tagged Snf1 or its catalytically inactive Snf1K84R counterpart. Figure 3A shows that only cells containing the active version of Snf1 were able to grow on ethanol or galactose as the sole carbon source. We isolated Snf1 complexes from these strains (Figure 3B) and tested their activity towards the Msn2-NLS *in vitro*. GST-extended Msn2 truncations were purified and used as substrate in a kinase assay with [γ -³²P]ATP. Figure 3C clearly shows that Snf1 can specifically phosphorylate the Msn2-S582. The control with purified inactive Snf1K84R kinase demonstrates that the phosphorylation of the S582 was solely due to the activity of Snf1. Experiments with purified PKA illustrated the fact that PKA could phosphorylate all four serines of the NLS, namely, S582, S620, S625, and S633. Only those constructs with the serines substituted to alanines lacked the appropriate phosphorylation signals.

A role for PP1 in the regulation of Msn2 activity

Our *in vivo* observations in *reg1* and *glc7T152K* cells suggest a dual role for PP1 in the regulation of Msn2: (1) indirect via regulation of the Snf1 kinase complex and (2) direct via dephosphorylation of Msn2. As in *S.c.* the gene *GLC7* is

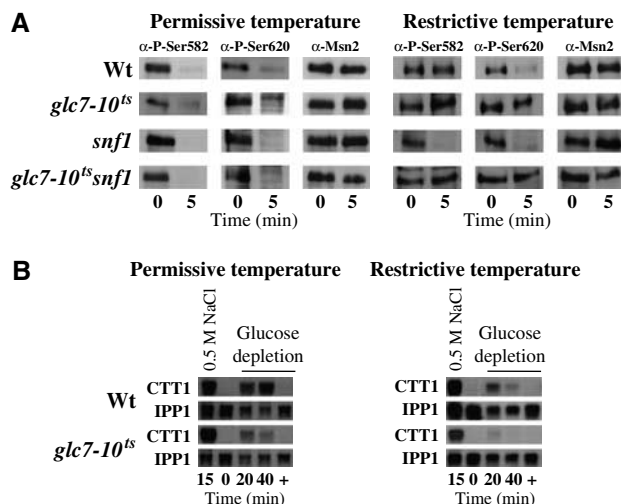


Figure 4 Glc7 is necessary for the dephosphorylation of the Msn2-NLS upon acute glucose depletion. (A) Inactivation of Glc7 prevents dephosphorylation of the Msn2-NLS upon acute glucose depletion. Wild-type (wt), *glc7-10^{ts}*, *snf1*-, and *glc7-10^{ts}snf1*-mutant cells were grown to OD_{600 nm} = 0.6. Then, cultures were split and each half incubated at permissive (26°C) or restrictive (37°C) temperature for 2.5 divisions. Samples were taken before (0) and 5 min after (5) acute glucose depletion. Western analyses of crude protein extracts were performed as described in Figure 1. (B) Total RNA was analyzed simultaneously for the expression of *CTT1* and *IPP1* as in Figure 2.

essential, we chose to work with a temperature-sensitive mutant strain (Andrews and Stark, 2000). Our results demonstrate that in *glc7-10^{ts}* cells the Msn2-NLS did not become dephosphorylated upon glucose depletion at restrictive temperature (Figure 4A). This effect seems to be independent of the presence of Snf1, since similar results were observed in *glc7-10^{ts}snf1* cells. In wild-type cells at restrictive temperature, Snf1 activity may mask the dephosphorylation of S582. We also tested the effect of Glc7 inactivation on the expression of *CTT1*, demonstrating that upon glucose depletion Msn2 transcriptional activity is greatly affected by the inactivation of Glc7 (Figure 4B).

In an attempt to determine whether the Msn2 dephosphorylation defects in *glc7-10^{ts}* cells were due to a direct interaction or due to a more indirect effect caused by a general change in cell physiology, we established an *in vitro* phosphorylation/dephosphorylation assay. We obtained the Msn2 substrates as bacterially expressed GST fusions. These proteins were radioactively labeled with affinity-purified yeast PKA. For GST-Msn2_(AA576-704), most of the phosphate groups are targeted to the four PKA sites within the NLS because a mutant product collectively affecting these sites remained virtually unphosphorylated (Görner *et al*, 2002). Enzyme assays were performed with PP1 phosphatase pulled down from *glc7* cells expressing a PrA-tagged version of Glc7 or wild-type cells expressing the PrA-tag only. The phosphorylated substrates GST-Msn2_(AA576-704), GST-Msn2, and calf histone were monitored for loss of radioactivity (Figure 5A). Control experiments showed that PKA-phosphorylated calf histone was not a substrate for PP1, while λ-phosphatase removed the modifications in all substrates. As shown in Figure 5A, the purified Glc7 complex can dephosphorylate both Msn2 substrates *in vitro*. However, a large amount of residual radioactivity remained in phospho-labeled full-

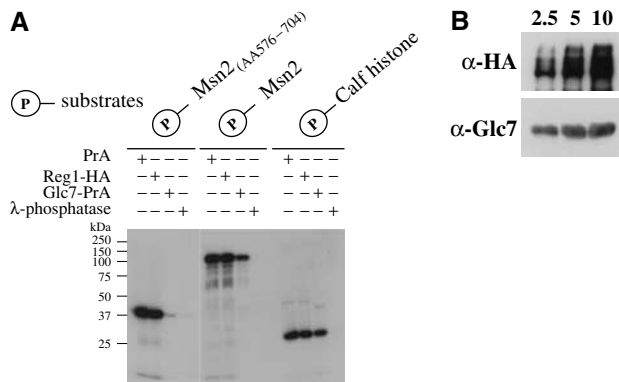


Figure 5 (A) Phosphorylated Msn2-NLS is substrate for Glc7 *in vitro*. Purified substrates GST-Msn2_(AA576-704), GST-Msn2, and calf histone were first phosphorylated *in vitro* with yeast PKA and subsequently incubated with excess of affinity-purified PrA, Reg1-HA, Glc7-PrA, or with λ -phosphatase. Samples were separated by SDS-PAGE and remaining phosphorylation was determined by autoradiography. (B) Reg1-HA complex contains Glc7. Western analysis of increasing amounts of Reg1-HA-attached beads, 2.5, 5, and 10 μ l, for the presence of Reg1-HA and Glc7.

length Msn2. The discrepancy observed between both Msn2 substrates in dephosphorylation efficiency reveals that Msn2 may contain sites that are nonspecifically phosphorylated by PKA, that Glc7 action on Msn2 is specifically targeted towards the NLS, or that the full-length protein is generally less recognized as substrate. Definitely, when we used the full-length GST-Msn2 as a substrate, we observed that PKA phosphorylated more sites than the strict consensus motifs proposed by Görner *et al* (2002). Incubation of phospho-Msn2 with a protein complex pulled down via the Reg1 subunit (Reg1-HA) did not lead to variations in the intensity of Msn2 phosphorylation even though the complex contained Glc7 (Figure 5B) and was positive in a phosphatase activity assay on myelin basic protein (data not shown). Together with our *in vivo* analysis (Figure 4A and B), these data fully support our contention that Reg1-independent PP1 activity is specifically directed against the Msn2-NLS.

A direct role for Snf1 in stress adaptation

The involvement of Snf1 in Msn2 regulation would explain that in *reg1* cells Msn2 is unable to activate STRE-gene transcription upon glucose depletion (Mayordomo *et al*, 2002). We therefore studied the phosphorylation pattern of Msn2 both in *snf1* and in *reg1snf1* cells. In wild-type cells, the phosphorylated serines of the NES (S288) and NLS (S582 and S620) are rapidly dephosphorylated and become rephosphorylated by an unknown kinase about 15 min after glucose depletion. Figure 6A shows that phosphorylation of S288 is still regulated in the mutants. In contrast, rephosphorylation of the NLS is greatly delayed after longer glucose depletion in *snf1*-mutant cells. This observation supports a crucial role for Snf1 in the inactivation of Msn2-NLS on S582, which would be followed by the action of PKA on S620 and the consequent adaptation of the cells to the absence of glucose. When glucose was added to wild-type cells, Msn2 was rapidly exported from the nucleus and rephosphorylated probably by the reactivated PKA, which seems to be partially able to phosphorylate S582. This hypothesis is confirmed by the analysis of the double mutant *reg1snf1*, in which the Msn2-NLS remained dephosphorylated until glucose was added to

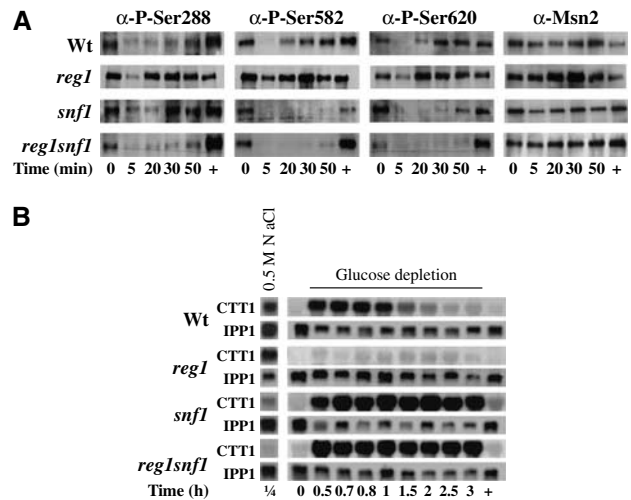


Figure 6 Snf1 is required for the inactivation of Msn2 during prolonged glucose depletion. (A) Snf1 is needed to rephosphorylate the Msn2-NLS during glucose depletion. Western blot analyses were performed with crude protein extracts obtained from wild type (wt), *reg1*-, *snf1*-, and *reg1snf1*-mutant cells before (0) and during acute glucose depletion (5, 20, 30, 50) and 20 min after glucose re-addition (+). Extracts were analyzed as described in Figure 1, with the additional use of α -P-S288 antibodies. (B) Snf1 is required to inactivate STRE-driven gene expression during prolonged glucose depletion. Wt, *reg1*-, *snf1*-, and *reg1snf1*-mutant cells were grown to mid-exponential phase (0) and either treated with 0.5 M NaCl for 15 min (1/4) or subjected to acute glucose depletion for the indicated time points, followed by immediate re-addition of glucose with the final sample taken after 20 min (+). Total RNA was analyzed simultaneously for the expression of *CTT1* and *IPP1*.

the cells. In order to confirm our assumption on Msn2 activity, we analysed the expression of *CTT1*. In wild-type cells, *CTT1* transcripts were detectable 30 min after glucose depletion for about 2 h, whereas in *reg1* cells no *CTT1* transcript could be detected. In *snf1* cells, expression of the *CTT1* reporter gene did not cease even 3 h later (Figure 6B). However, when glucose was added, all responding cells stopped to transcribe *CTT1*. This demonstrates the involvement of two independent regulation mechanisms to inactivate Msn2: (1) an Snf1-dependent mechanism in the absence of glucose and (2) an Snf1-independent process in the presence of glucose (Figure 6B). The latter is most likely attributed to a constant PKA activity in glucose-growing cells.

To test whether Snf1 activity is responsible for the lack of Msn2 activity in *reg1* cells, we also analyzed the *CTT1* expression levels in *reg1snf1* cells. In the absence of *REG1*, Glc7 cannot act on Snf1; therefore, the kinase is constitutively active. With the additional absence of *SNF1*, Msn2 should not be instantaneously inactivated; consequently, the effect of the *REG1* deletion should be directly visualized on *CTT1* expression pattern. Indeed, our Northern analyses corroborate this proposition, while no *CTT1* expression can be detected in *reg1* cells; *reg1snf1* cells present a 2.5-fold higher expression than wild-type cells under the same conditions (Figure 6B). It is noteworthy that *reg1snf1* cells are unresponsive to stress, as shown by the absence of *CTT1* transcript upon osmotic shock.

Snf1 initiates adaptation to the lack of glucose at the Msn2-NLS level

To illustrate whether Snf1 is required for Msn2 cytoplasmic relocalization under prolonged glucose starvation, we

followed the location of Msn2-GFP *in vivo*. To distinguish *msn2msn4* from *msn2msn4snf1* cells, we additionally expressed the DsRed fused mitochondrial protein ATPase subunit 9 only in *msn2msn4* cells. We mixed both cell cultures just before observation and directly followed their fluorescence. Figure 7A shows that about 150 min after glucose depletion cells with DsRed labeling had relocated their Msn2-GFP to the cytoplasm, whereas in *snf1* mutants GFP remained fully nuclear. The observation demonstrates that Snf1 function is important for the decrease of Msn2 nuclear import rates. We therefore asked whether single amino-acid substitutions in Msn2 at position 582 could mimic the absence or presence of Snf1 activity. We expressed Msn2-S582A and Msn2-S582D in *msn2msn4* cells and followed phosphorylations at S288 and S620, as well as the transcriptional activity of Msn2. As shown in Figure 7B and C, the status of S582 has a considerable influence on the overall phosphorylation pattern and function of Msn2. In the context of S582A, which mimics a dephosphorylated site, Msn2-S620 rephosphorylation was highly retarded, whereas, in the context of S582D, Msn2-S620 was prematurely rephosphorylated. To confirm that Msn2 activity was impaired by these mutations, we also followed the expression of *CTT1* in the depicted strains. As predicted, cells expressing Msn2-S582A produced higher *CTT1* transcript levels than cells expressing Msn2, whereas cells expressing Msn2-S582D produced much lower *CTT1* transcript levels. Our data strongly suggest that

the phosphorylation of S582 by Snf1 constitutes a priming event required for the full rephosphorylation of the Msn2-NLS and the decrease in Msn2 nuclear concentration.

Discussion

Since the level and turnover of the transcription factor Msn2 remain stable through short periods of stress and even through complex alterations in nutrient supply, most changes in Msn2 activity must be attributed to the regulation of either its post-translational modifications or its interactions with regulatory partners. Msn2 was already described to be hyperphosphorylated upon heat shock (Garreau *et al*, 2000) and rapidly dephosphorylated after acute glucose removal (Görner *et al*, 2002), indicating that different independent mechanisms participate in its regulation. Similarly, the function of PP2A was proposed to be essential to regulate Msn2 activity under stress conditions, but not under glucose depletion (Santhanam *et al*, 2004). In this study, we further investigated the influence of glucose signaling on the activation state of Msn2. As documented previously, PKA controls at least partially the activity of Msn2 via direct phosphorylation (Görner *et al*, 2002). Here we develop this theme in several important directions. Our results show that PP1 plays a crucial role in the regulation of Msn2 by directly antagonizing phosphorylation at consensus motifs that regulate its NLS. We could clarify the role of Snf1 in the downregulation of Msn2 during prolonged glucose starvation, as it modifies the S582, a site recognized by PKA during exponential growth with glucose. Finally, the results of our study may serve as a paradigm to describe the complexity of resolving a network of signaling communications in which both kinases and phosphatases participate in multiple regulatory interactions.

The previous contention that the inhibition of PKA was the main cause for Msn2 activation during glucose starvation was based on experiments with *bcy1* cells. These mutants seemed to have much less pronounced effects on phospho-Msn2 after glucose withdrawal than the corresponding wild-type cells (Görner *et al*, 2002). While repeating the analysis of *bcy1* cells, we observed a much less drastic effect on Msn2 regulation. The results presented here would suggest that glucose deprivation is either affecting the access and function of the PKA catalytic subunits towards their substrates, or that a sizeable part of Msn2-NLS dephosphorylation could ensue through an increase in specific phosphatase activity. The predominant regulation of a C₂H₂ zinc-finger transcription factor by a phosphatase is not without precedent in yeast. Crz1, a factor quite related to Msn2 with regard to its overall structure, is also modified and inhibited by PKA in its nuclear import domain. In this case, the activation of the Ca²⁺/calmodulin-dependent phosphatase calcineurin seems to exert the main control on the behavior of the transcription factor (Kafadar and Cyert, 2004).

The differences in results and conclusions between the study from Görner *et al* (2002) and ours might have a trivial cause. We suspect that their quantifications might have been distorted because their measurements were within or close to the non-linear range of Western analysis. For instance, in exponentially growing cells, the authors did not observe a distinct increase of Msn2-NLS phosphorylation in *bcy1* cells. In contrast, we have consistently measured a four- to five-fold increase in phosphorylation in *bcy1* cells compared to wild-type cells.

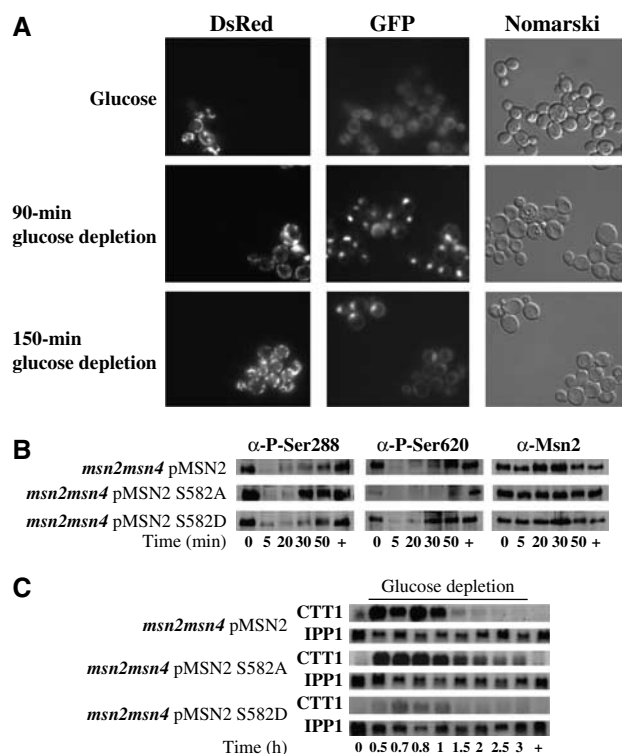


Figure 7 Snf1 is required for the export of Msn2 from the nucleus and its inactivation upon prolonged glucose depletion. (A) *msn2msn4*-mutant cells expressing ATPaseSU9-DsRed and Msn2-GFP and *msn2msn4snf1*-mutant cells expressing only Msn2-GFP were grown to midexponential phase and mixed while subjected to acute glucose depletion for the indicated time points. (B, C) *msn2msn4*-mutant cells expressing Msn2, Msn2-S582A, or Msn2-S582D were grown and treated as described in Figure 6, as were the subsequent Western (B) and Northern (C) analyses.

The assignment of a direct role for a protein phosphatase has been notoriously difficult. Assessments based on genetic data can be misleading due to the rather pleiotropic effects on the available mutants, while biochemical approaches can be hampered by either the loss of necessary adaptors for the substrate or a highly unstable behavior of the enzyme *in vitro*. These problems have been particularly notable for PP1 function, owing to the large number and variety of regulatory subunits. A role for PP1 in the regulation of Msn2 has already been projected by previous work (Mayordomo *et al*, 2002). However, in this study, the genetic evidence was obscured by the fact that Snf1 appeared as the main regulatory component affected by the loss of the PP1 subunit Reg1. Although *reg1* mutants presented a severe defect in Msn2 localization, *snf1reg1* double mutants behaved again like wild-type cells. Still, the authors never investigated whether the absence of Glc7 activity would mimic the *reg1*-mutant effects and if so whether these were suppressed by the loss of *SNF1*. Our studies show that, in contrast to *reg1snf1* mutants, in *glc7-10^{ts}snf1* mutants the Msn2-NLS still exhibits a strong de-phosphorylation defect in response to glucose starvation. Therefore, the reasonable assumption is that PP1 is indeed the phosphatase that antagonizes PKA activity at Msn2-NLS. It should be noted, however, that the Msn2 dephosphorylation defect in *glc7-10^{ts}* cells was difficult to document, first of all because it takes some time to inactivate the catalytic subunit and second because prolonged heat stress itself appears to eventually cause an increase in PKA and Snf1 kinase activities. Nevertheless, our data illustrate that the phosphatase must have a role that is independent of Reg1 and its substrate Snf1, a conclusion that was corroborated by our biochemical analysis. Immune purification of the protein complex using tagged Reg1 did not yield any material with phosphatase activity towards Msn2, whereas the complex purified via Glc7 could dephosphorylate its target (Figure 5). Thus, there should be little doubt that the phosphatase directly acts on these sites.

Bud14 has recently been proposed as putative Msn2-regulating factor during or after diauxic shift (Lenssen *et al*, 2005). However, our study shows that *bud14* cells had no defect during acute glucose depletion and we still do not know the regulatory subunits required for the recognition of Msn2. Since a large set of additional candidate mutants did not yield the expected phenotype (Supplementary Figures S1 and S2), it could be that lethality or redundancy prevented us from identifying such factors in a genetic approach. One can even envisage the existence of multiple docking sites with different recognition features for different PP1 complexes, possibly distributed throughout the whole Msn2 protein. Nevertheless, structures recognized by the phosphatase must be embedded within a 120 amino-acid domain containing the NLS- and DNA-binding domain, since PP1-dependent dephosphorylation is specific for such a fragment both *in vitro* and *in vivo*. In fact, the Msn2-NLS might be one of the first genuine targets that can be used as a specific *in vitro* substrate of PP1, perhaps opening a biochemical approach towards the identification of the regulatory subunits.

The effects of glucose starvation on the phosphorylation level of Msn2 are only transient (Figure 6). One can notice significant rephosphorylation of Msn2 about 20 min after glucose withdrawal, an effect that will eventually result in a decrease of stress-related gene expression. According to our

results, Snf1 must not only play a crucial role in this adaptive response but also a direct one. First, we demonstrate a correlation between the transcriptional response and Msn2-S582 dephosphorylation in *snf1* cells. Second, we show that Snf1 could directly phosphorylate Msn2-S582 *in vitro*. Yet, the consequences of S582 phosphorylation by Snf1 are quite dramatic as cells with high Snf1 activity lack any Msn2 response to glucose starvation (Mayordomo *et al*, 2002). We suspect that this result is due to a rather complete phosphorylation of the pool of Msn2 owing to a high affinity of Snf1 kinase for this specific site. At normal levels, PKA seems to lead to only a smaller amount of molecules carrying the S582 modification (about 20%) if we assume that *bcy1* mutants are close to saturation. Such a view is also supported by the fact that S582 phosphorylation levels are elevated to the same extent in *reg1* and *bcy1* mutants. Moreover, we observed that modification at S582 partly functions as a priming event for the phosphorylation of other PKA-dependent sites in Msn2-NLS. Subtle regulation of a transcription factor via post-translational modifications has already been described for Pho4 (Springer *et al*, 2003). Mutating specific sites within its localization domains, Springer *et al* (2003) could point out a negative correlation between the phosphorylation state of Pho4 and its binding capacity to promoters of the Pho-regulon. Conceptually similar to Pho4 regulation, differential phosphorylation of Msn2 could lead to activation of different subsets of STRE-containing genes, especially since the Msn2-dependent expression of a given gene differs according to environmental conditions (Amoros and Estruch, 2001).

Several recent observations seemed to connect the function of Snf1 kinase with PKA activity and stress signals particularly in cells under nutrient limitation, salt exposure and heat shock (Sanz, 2003; Hahn and Thiele, 2004; Hedbacker *et al*, 2004). A mutant analysis demonstrated that the absence of PKA activity resulted in the relocalization of some Snf1 kinase complexes, thereby raising the possibility that the PKA pathway participated in the regulation of Snf1 (Hedbacker *et al*, 2004). DNA microarray studies documented that about 30% of the heat shock factor (HSF) target genes are also induced by the diauxic shift. The authors showed that Snf1 directly phosphorylated HSF, and using chromatin immunoprecipitation they demonstrated that this phosphorylation event enhanced chromosomal HSF DNA binding to low-affinity target promoters, for example, *SSA3* and *HSP30* (Hahn and Thiele, 2004). Although we found some unexpected evidence for a positive role of Snf1 in Msn2-driven transcription upon osmotic shock, the mechanism and physiological relevance for this effect await further investigations. More importantly, in our study, we could clearly document an adaptive function for Snf1 kinase with regard to the general stress response. From classical genetic studies of the PKA pathway as well as the use of Msn2 hyperactive alleles, it has become apparent that a prolonged stress response has a rather detrimental effect on cellular growth and proliferation. To liberate the cells from growth arrest, several mechanisms have been proposed for the downregulation of Msn2. One study favored the view that the export rate of Msn2 might be increased by Srb10-induced phosphorylation (Chi *et al*, 2001). Other groups rather contented that higher degradation rates of Msn2 in the nucleus were responsible for adaptation (Durchschlag *et al*, 2004; Lallet *et al*, 2004; Bose *et al*, 2005). Here we add yet another adaptation

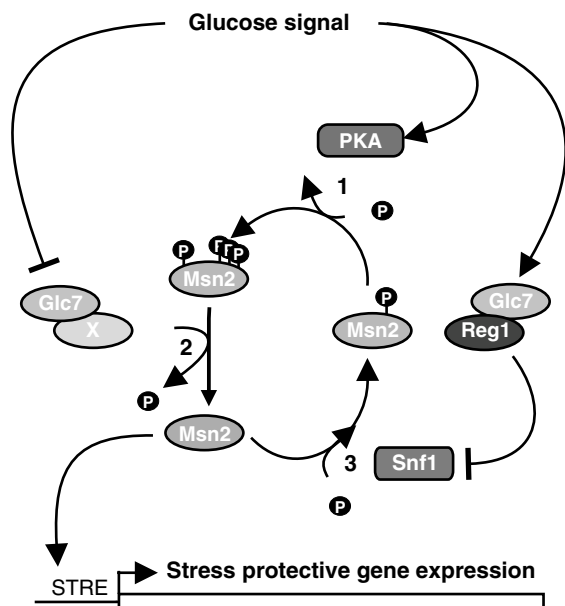


Figure 8 A switch diagram for the regulation of Msn2 upon acute glucose depletion. (1) Msn2 is phosphorylated and inactivated by PKA during growth on glucose, while the Snf1 kinase is kept in an inactive state by PP1 (Glc7-Reg1). (2) Glucose depletion results in Msn2 dephosphorylation by PP1-Glc7 associated with unknown regulatory subunit(s), followed by Msn2 nuclear accumulation and activation of STRE-driven genes. It also causes the rapid activation of Snf1 kinase. (3) Cells adapt to the loss of glucose as the rising level of Snf1 kinase throttles nuclear entry of Msn2.

mechanism that is specifically geared towards the situation after glucose deprivation. It relies on the direct phosphorylation of Msn2 by Snf1, a modification that may attenuate its nuclear import. Our current model (Figure 8) summarizes the complicated interplay between PP1 and the different protein kinases for the regulation of Msn2 function during acute glucose fluctuations.

Materials and methods

Strains, plasmids, growth conditions, immunological and Northern blotting

Supplementary data contain detailed information on the strains (Supplementary Figures S1 and S2), plasmids, and growth conditions used in this study, and a description of the applied Northern and Western blot analysis procedures.

GST purification

Escherichia coli (DH5 α) cells transformed with plasmids coding for GST-extended proteins were grown to mid-exponential phase in 50 ml 2 \times YT and induced with 1 mM IPTG for 4 h. GST substrates were isolated using MicroSpin GST columns (Amersham) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE and visualized by silver staining.

Yeast PKA purification

The purification of yeast PKA using a *bcy1* mutant expressing a TAP-tagged version of Bcy1 was described before (Görner *et al*, 2002).

Snf1 purification

snf1-mutant cells expressing either pHA-Snf1 or pHA-Snf1K84R were grown in 500 ml YPD (1.2×10^7 cells/ml), harvested at 4000 g for 5 min, washed twice with water and resuspended in 5 ml ice-cold lysis buffer (IPP150, complete protease inhibitor cocktail EDTA-free (Roche), 2 mM PMSF). Cells were lysed with glass beads and centrifuged at 5000 g for 10 min (4°C). Lysates were clarified further

by centrifugation at 10 000 g for 10 min (4°C) and incubation on a rotator for 12–14 h (4°C) with PanMouse IgG Dynabeads (DYNAL Biotech), chemically crosslinked (DMP-Sigma) with culture medium. Cleared lysates were incubated for 6 h on a rotator (4°C) with PanMouse IgG Dynabeads crosslinked with C12A5 hybridoma supernatant (α -HA). Subsequently, beads were collected by centrifugation (4°C), washed twice in 2 vol. IPP150, twice in 2 vol. washing buffer (IPP150, 0.5 mM EDTA, and 1 mM DTT) and resuspended in 400 μ l Snf1-kinase buffer (20 mM HEPES, pH 7.0; 0.5 mM EDTA, pH 8.0; 0.5 mM DTT; 5 mM Mg-acetate) containing 10% glycerol ready for storage.

Kinase assays

For each reaction, 10 μ g of purified GST-Msn2 substrate was incubated with [γ - 32 P]ATP (5 μ Ci, 3000 Ci/mmol) and purified PKA, Snf1, or Snf1K84R for 30 min (30°C) in either PKA-elution buffer supplemented with 100 mM Tris, pH 7.4 and 2 mM MgCl $_2$, or in Snf1-kinase buffer, respectively. Simultaneously, control assays were performed by omitting the respective purified kinase from the reaction. Reactions were terminated by the addition of an equal volume of preheated SDS-loading buffer and boiled for 10 min. Proteins were separated by SDS-PAGE, loadings were analyzed by silver staining, and incorporation of radioactive phospho-groups visualized by autoradiography.

PP1 assay

GST-Msn2 fusions and Calf Histone (Calbiotech) were phosphorylated *in vitro* with PKA. Phosphorylated substrates were separated by SDS-PAGE, eluted from the gel, and the amount of incorporated [γ - 32 P]ATP was quantified (Cerenkov). Each phosphatase assay was initiated with 1500 c.p.m. of phosphorylated substrate. Purification of Reg1-HA was performed as described for Snf1-HA. TAP purifications were carried out simultaneously from the yeast strains PWY3 and LKY150 using the same amounts of total proteins and IgG-Sepharose beads, according to Walsh *et al* (2002). Briefly, cells were grown in 500 ml YPD (1.2×10^7 cells/ml), harvested at 4000 g for 5 min and shock-frozen. Pellets were resuspended in 5 ml of lysis buffer (100 mM KCl, 0.1% Triton X-100, 0.1 mM EDTA, pH 8.0, 1 mM MgCl $_2$, 50 mM HEPES-KOH, pH 7.5), supplemented with complete protease inhibitors (Roche). Cell suspensions were lysed with glass beads, centrifuged at 5000 g for 20 min, and cleared further by centrifugation for 30 min at 15 000 g. Cleared lysates were used immediately for overnight incubation (4°C), each with 500 μ l IgG-Sepharose beads, washed three times in lysis buffer. The phosphatase assays were performed with 25 μ l PrA-TEV-bound beads, PrA-TEV-Glc7-bound beads, Reg1-HA-bound beads, or 1 μ l λ -phosphatase on phosphorylated GST-Msn2_(AA576-704), GST-Msn2, and calf histone. Aliquots were analyzed for the presence of Glc7 by Western blot.

Fluorescence microscopy

Glucose depletion was performed as described in Supplementary data, and cells were visualized live unfixed. Cells were visualized with an $\times 63$ objective from a Zeiss Axioplan 2 fluorescence microscope and images were collected with a Quantix CCD camera using the Lightview software and processed in Adobe Photoshop 8.0.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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