A shared regulatory network allows functional coupling of Pho89 and Ena1 in response to environmental alkalinization

Albert Serra Cardona

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Universitat Autònoma de Barcelona

ibb Institut de Biotecnologia i de Biomedicina

RESULTS AND DISCUSSION

1. Analysis of the promoter region of *PHO89*

Pho89 was characterized for the first time more than 15 years ago (Martinez and Persson, 1998). In that work, the authors identified Pho89 as the protein responsible for the previously described sodium-phosphate cotransport (Roomans *et al.*, 1977) and found that the *PHO89* promoter contained putative Pho4-binding sites. Later, *PHO89* was described to be upregulated in the presence of $CaCl_2$ in a calcineurin-dependent manner (Yoshimoto *et al.*, 2002), and Serrano and coworkers reported that this gene was strongly induced by alkaline pH stress by the calcineurin pathway through the Crz1 transcription factor (Serrano *et al.*, 2002). In order to further characterize the alkali-regulation of *PHO89* we decided to analyze its promoter region to search for the putative Pho4- and Crz1-binding sites. In addition, we decided to include in the search possible regulatory sites recognized by other elements related to the alkaline stress response. Since *ENA1* was shown to exhibit induction kinetics similar to *PHO89* in alkaline stress (Serrano *et al.*, 2002), we thought interesting to include in the analysis sequences associated with repressors Nrg1/Nrg2 and Mig1/Mig2, previously found to regulate the transcription of *ENA1* in high pH conditions (Platara *et al.*, 2006). Moreover, we also decided to search for STREs, recognized by the general stress response transcription factors Msn2/Msn4, which are major regulators of the short-time response to alkaline stress (Casado *et al.*, 2011).

As shown in table 3, the *PHO89* promoter contains several putative Pho4-binding sites, one of them with a high probability (situated at position -324 to -317 nt from the initiating Met codon). As expected, we also found possible CDREs (recognized by Crz1), two of them (denoted in bold) with a high probability. Interestingly, our search also produced putative sites known to be recognized by repressors Nrg1/Nrg2 and Mig1/Mig2, suggesting that *PHO89* may be under the control of additional factors apart from Pho4 and Crz1. It is worth mentioning that no Msn2/Msn4-binding sites were identified on this promoter, probably indicating that this gene would not be affected by the general stress response.

Table 3. *In silico* analysis of the *PHO89* promoter.

Factor	start	end	sequence	weight	*P* value
Crz1	-620	-612	CGAAGCCAG	6.1	4.8e-04
Mig1/2	-584	-578	CACCCGC	4.5	8.3e-04
Crz1	-555	-547	TCGCGCCAC	6.8	2.8e-04
Crz1	**-513**	**-505**	**CACAGCCAC**	**8.6**	**2.0e-05**
Mig1/2	**-466**	**-460**	**CCCCACG**	**8.4**	**7.5e-05**
Pho4	-464	-457	CAACGTGG	7.3	1.6e-04
Nrg1/2	**-440**	**-429**	**TTAAGGGCCCAC**	**8.8**	**1.3e-05**
Pho4	**-324**	**-317**	**CCACGTGC**	**10.3**	**1.3e-05**
Crz1	**-275**	**-267**	**CACAGCCAC**	**8.6**	**2.0e-05**
Nrg1/2	-265	-254	AGAAGGATCCAA	0.4	9.4e-04
Crz1	-166	-158	CTAAGCCCG	6.4	3.8e-04

Nucleotides from positions -800 to -1 (from the initiating Met codon) of *PHO89* were analyzed with the matrix-scan algorithm available at the RSAT website (Thomas-Chollier *et al.*, 2011) using a *P* value threshold of 0.001. Matrices for transcription factor binding sequences were obtained from the JASPAR database (Portales-Casamar *et al.*, 2010).

2. Expression profiles of Pho89 and Pho84: a comparative analysis

Pho84 and Pho89 constitute the high-affinity phosphate transport system in *S. cerevisiae* and they have been described as members of the *PHO* regulon, i.e. inducible under phosphate starvation conditions in a Pho4-dependent manner (Bun-Ya *et al.*, 1991; Martinez and Persson, 1998). However, as mentioned in section 4.2 of Introduction, there are some differences in their regulation, being perhaps the most striking the fact that *PHO89* displays an early alkaline induction, whereas *PHO84* expression after high pH stress occurs later (Serrano *et al.*, 2002).

Taking this into account, our purpose was to determine quantitatively the contribution of Pho4 to the alkaline induction of *PHO89*. In addition, since high pH stress provokes a response that mimics a phosphate starvation situation, we thought it would be interesting to compare the kinetics between Pho84 and Pho89 expression when cells are subjected to both types of stress.

2.1. Pho89 is rapidly induced by high pH stress and upregulated by Pho4

To assess the contribution of Pho4 to *PHO89* mRNA levels after high pH stress we performed a RT-PCR experiment over a time course with wild type and *pho4* mutant strains. As shown in figure 7a, the wild type strain growing at standard conditions (YPD at pH 5.5) displays no detectable signal for *PHO89*. In contrast, 5 minutes after alkaline stress at pH 8.0, *PHO89* mRNA levels markedly increase and, from this point, the levels decrease until 40 minutes, when the signal is barely visible. Thus, the alkaline induction of *PHO89* is fast but transient, with its peak around 5 minutes after the stress. This is in accordance with what was previously reported in Serrano *et al.* 2002, where the authors detected by northern blot the presence of *PHO89* mRNA also 5 minutes after high pH stress. In that time course, however, *PHO89* expression declined faster, probably due to the milder alkaline stress employed (pH 7.6 vs. pH 8). In our case, RT-PCR analysis performed in a *pho4* mutant strain showed an impaired expression of *PHO89*: the signal is only visible at time points 5 and 20 minutes and with much lower intensity.

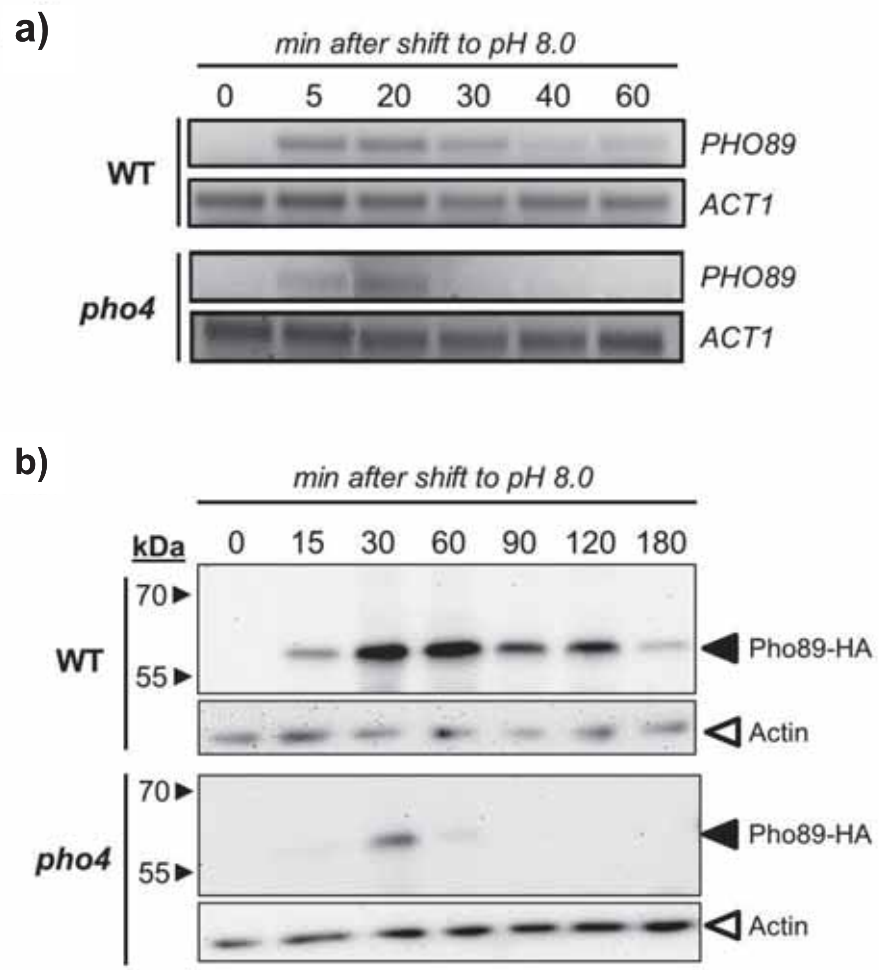

Figure 7. **a)** Wild type DBY746 and RSC4 (*pho4*) cells were collected at the indicated times after switching the medium to pH 8.0 and total RNA prepared. Semi-quantitative RT-PCR was carried out using oligonucleotides specific for *PHO89* and *ACT1* (as control) and the products were resolved in agarose gels and stained with GelRed™ (Biotium Inc.). **b)** Strains ASC07 (*PHO4 PHO89*:3xHA) and ASC10 (*pho4*::LEU2 *PHO89*:3xHA) were grown on YPD until OD_{660} 0.6. Cultures were then shifted to pH 8.0 and growth was resumed. Samples were taken at the indicated times and processed for protein extract preparation as indicated in Experimental Procedures. Equivalent amounts (30 µg of total protein) were subjected to SDS-PAGE (10% polyacrylamide gels) followed by immunoblot using anti-HA antibodies to reveal HA-tagged Pho89 (dark triangles). Membranes were stripped and reprobed with anti-actin antibodies (open triangles) for loading and transfer reference.

In order to detect Pho89 protein accumulation under similar conditions, a C-terminally HA-tagged version of Pho89 was introduced into its own chromosomal locus in wild type and *pho4* strains. In the wild type strain, Pho89 is already visible 15 minutes after alkaline stress, with maximum accumulation at 30 minutes (figure 7b). From there, Pho89 levels decrease until 180 minutes, when it is hardly detectable. Equivalent to the RT-PCR experiment, the *pho4* strain shows overall reduced levels of Pho89, only detectable at 30 and 60 minutes after alkaline stress. This confirms that, even though Pho4 plays an important role in *PHO89* alkaline induction, there are still other factors contributing to its expression.

2.2. Pho84 and Pho89 display remarkable differences in their expression under low P_i or alkaline conditions

After assessing Pho89 expression over time under high pH stress we wondered what would be the differences in its accumulation between subjecting cells to alkaline stress or phosphate scarcity. Under phosphate starvation conditions, Pho89 accumulated slowly, and was detectable only 4 and 8 hours after the shift to low P_i medium (figure 8a). As expected, this response was largely abolished in a *pho4* mutant. In contrast, high pH stress in the high P_i medium yielded an early but transient expression of Pho89, with its peak at 30 and 60 minutes after the alkaline induction. Interestingly, when cells were exposed to both phosphate scarcity and alkaline pH, Pho89 showed an accumulation pattern that was the combination of that observed for each individual stress: a fast and transient response followed by a late accumulation.

In order to compare the Pho89 and Pho84 expression kinetics in low Pi or high pH conditions, we performed the same experiment but detecting Pho84, the other high-affinity phosphate transporter. As shown in figure 8b, Pho84 accumulation after the shift to alkaline pH occurs later than that observed for Pho89, appearing 60 minutes after the stress and reaching its maximum at 180 minutes. Curiously, Pho84 expression in response to phosphate starvation is essentially identical to the response to high pH stress, although slightly stronger. It is worth noting that, in contrast to that observed for Pho89, in both cases Pho84 induction is fully dependent on Pho4, as deduced by the total absence of signal in the *pho4* strain.

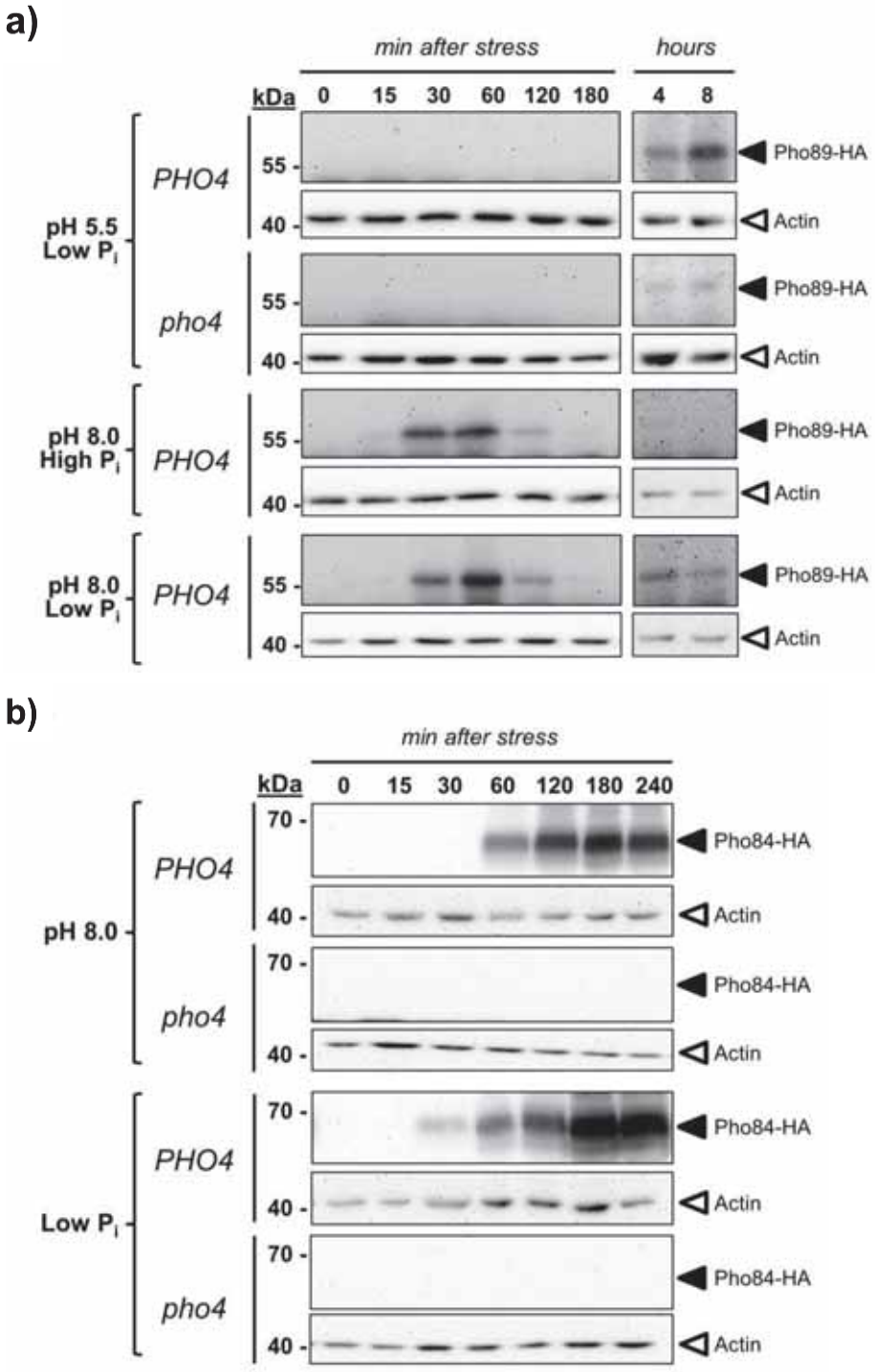

Figure 8. a) Strains ASC07 (*PHO4 PHO89*:3xHA) and ASC10 (*pho4*::LEU2 *PHO89*:3xHA) were grown on synthetic high P_i medium until OD_{660} 0.6. Cells were collected and resuspended in synthetic medium lacking phosphate, supplemented with 10 mM P_i (high P_i) or 100 µM P_i (low P_i) at pH 5.5 or/and pH 8.0, adjusted with KOH. Growth was resumed and samples were taken at the indicated times and processed for immunoblot as described in the legend of figure 7b. **b)** Wild type strain DBY746 and its *pho4* derivative (RSC4) were transformed with the centromeric plasmid pMM15-PHO84, which expresses the C-terminally HA-tagged version of Pho84. Cells were subjected to high pH or low phosphate stress, collected after the indicated periods and the amount of Pho84 assessed using anti-HA antibodies. Correct loading and transfer was monitored with anti-actin antibodies in the stripped membranes.

These results demonstrate the differences between Pho89 and Pho84 regulation. As previously reported, both transporters are induced under phosphate starvation conditions in a Pho4-dependent fashion (Ogawa *et al.*, 2000); however, we show that Pho84 displays a greater and faster accumulation than Pho89 when phosphate is scarce. This correlates with the transcriptional profile described in Zvyagilskaya *et al.*, 2008, where the authors showed that *PHO89* was poorly induced in a low phosphate medium. On the other hand, Pho89 expression after alkaline stress occurs earlier, in accordance with the results described by Serrano *et al.*, 2002. Differences in the kinetics of expression of *PHO84* and *PHO89* have been described for other stresses. For example, in the presence of the cell-wall damaging agent Congo red, *PHO89* response is faster than that of *PHO84* (Garcia *et al.*, 2004), and cells exposed to Mg^{2+} deprivation induce *PHO89* but not *PHO84* (Wiesenberger *et al.*, 2007). In contrast, a recent work stated that potassium deprivation upregulates *PHO84* but not *PHO89* (Barreto *et al.*, 2012). Therefore, even though both transporters belong to the *PHO* regulon, the differences in their regulation are evident and probably caused by factors impinging on their promoters. For instance, Pho84 upregulation is very complex, involving antisense RNAs and chromatin remodeling (see section 3.2 of Introduction), but Pho4 seems to be the only transcription factor directly upregulating its expression. On the other hand, Pho89 appears to be regulated by additional elements, which would account for its faster response under certain conditions.

3. The role of the calcineurin pathway in *PHO89* alkaline induction

DNA microarray data from studies focused in the transcriptomic changes in response to alkaline pH or calcium identified *PHO89* as a possible target for the calcineurin pathway (Serrano *et al.*, 2002; Viladevall *et al.*, 2004; Yoshimoto *et al.*, 2002). Both situations trigger a fast burst of cytosolic calcium, which binds to calmodulin and, in turn, activates calcineurin (see section 2.2 of Introduction). Although Crz1 is the major factor involved in the calcineurin-mediated response, the phosphatase has other substrates that also participate in this response.

To further investigate the mechanism by which calcineurin upregulates *PHO89*, we thought it would be interesting to measure Pho89 mRNA and protein levels in a strain lacking calcineurin activity. We also wanted to assess the role of Crz1 in this induction and to characterize its possible binding to the promoter of *PHO89*.

3.1. The alkaline induction of Pho89 is strongly dependent on calcineurin

To evaluate the effect of calcineurin on the alkaline induction of *PHO89* we followed by RT-PCR its mRNA accumulation over time in a strain lacking *CNB1*, encoding the regulatory subunit of calcineurin.

As shown in figure 9a, *PHO89* mRNA accumulation is barely noticeable in the *cnb1* strain: only slight amplification bands can be seen at 5 and 20 minutes after stress, coinciding with maximum accumulation in the wild strain. To evaluate if this effect could be extrapolated to Pho89 protein expression, we carried out immunoblot analysis with the Pho89 HA-tagged wild type and *cnb1* strains. As shown in figure 9b, the *cnb1* strain exhibits a severe impairment in Pho89 expression, in accordance to what we observed by RT-PCR. It is worth noting that even though *pho4* and *cnb1* mutations have a negative effect on Pho89 mRNA and protein levels, the lack of calcineurin activity seems to have a stronger impact on the upregulation of the transporter.

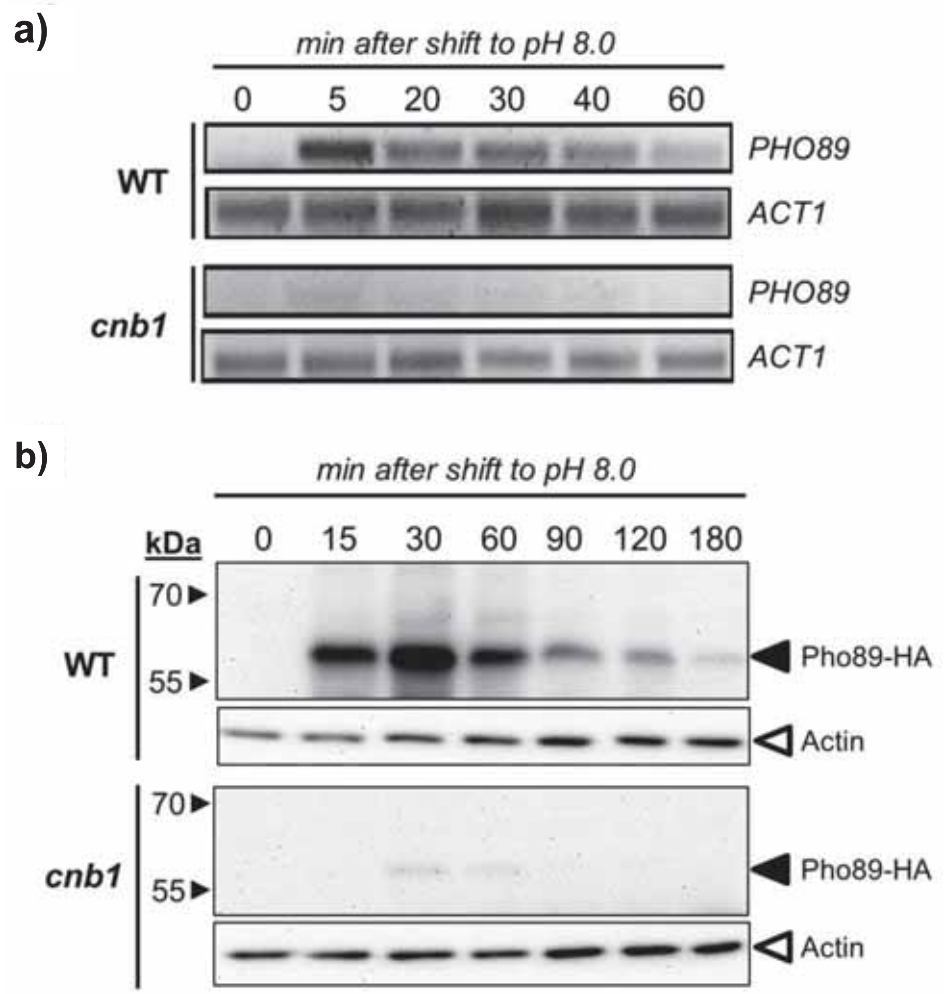

Figure 9. a) Wild type DBY746 and MAR15 (*cnb1*) cells were collected at the indicated times after switching the medium to pH 8.0 and total RNA prepared. Semi-quantitative RT-PCR was performed as described in the legend of Figure 7a. **b)** Cultures of strains ASC07 (*PHO89:3xHA:HIS3*) and ASC08 (*cnb1 PHO89:3xHA:HIS3*) were shifted to pH 8.0 and protein extracts were prepared. Samples (30 μg of protein) were resolved by SDS-PAGE and processed for immunoblot as described in the legend of Figure 7b.

3.2. The calcineurin pathway upregulates *PHO89* through Crz1 binding to its promoter

The alkaline expression of *PHO89* was reported to be reliant on the Crz1 transcription factor (Serrano *et al.*, 2002). Since calcineurin dephosphorylates multiple targets apart from Crz1, affecting several cellular processes, we wanted to investigate if its effect on *PHO89* alkaline induction depended exclusively on Crz1. For that, we monitored the activation after high pH stress of the *PHO89* promoter fused to a *lacZ* reporter gene (pPHO89-LacZ) in wild type, *cnb1*, *crz1*, and *cnb1 crz1* strains. As shown in figure 10a, lack of the phosphatase, its downstream transcription factor, or both components resulted in a

strong decrease in the induction of the promoter, practically identical in all cases. This suggests that the calcineurin-mediated alkaline induction of *PHO89* depends solely on Crz1, and not on other targets of the phosphatase.

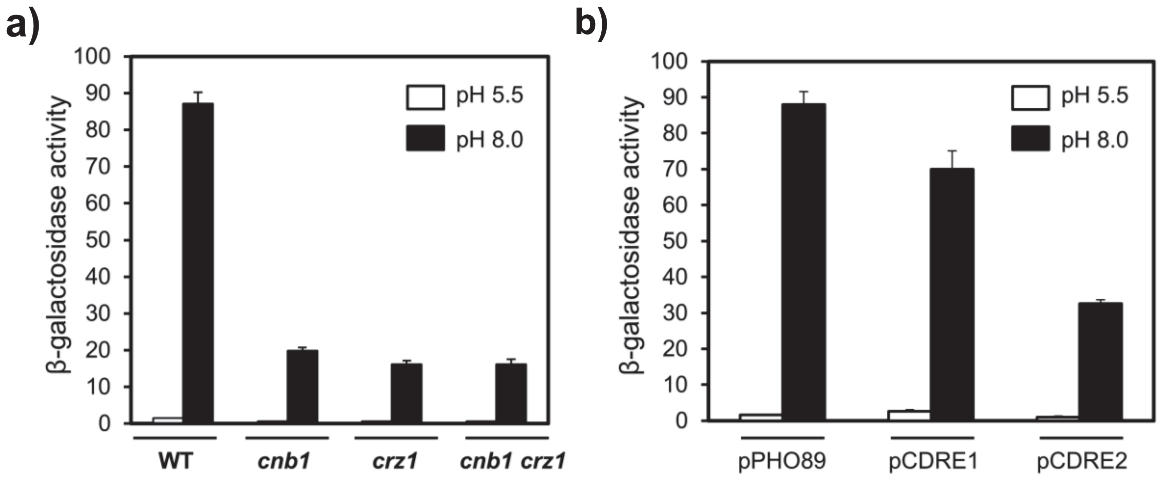

Figure 10. a) The indicated strains were transformed with the reporter pPHO89-LacZ and exponentially growing cultures were exposed to pH 8.0 (closed bars) or maintained at pH 5.5 (empty bars) for 90 min. Cells were then collected and β-galactosidase activity measured as indicated in Experimental Procedures. Data are mean ± SEM from 12 determinations. **b)** Wild type strain DBY746 was transformed with plasmid pPHO89-LacZ, pPHO89^{CDRE1}-LacZ or pPHO89^{CDRE2}-LacZ and cultures subjected to pH 8.0 (closed bars) or maintained at pH 5.5 (empty bars). Data are mean ± SEM from 12 determinations.

The dephosphorylation of Crz1 by calcineurin prompts its nuclear accumulation and thereby its binding to specific sequences known as CDREs. As depicted in table 3, the promoter of *PHO89* contains two putative CDRE binding sites: the upstream sequence, termed CDRE1 (GTGGCTG) and located at positions -513 to -507 upstream of the initiating Met codon, and the downstream sequence, termed CDRE2 (CAGCCAC) and located at positions -273 to -267. In order to determine the functionality of both elements we modified their GCC core characteristic of the CDREs, thus changing CDRE1 to GTG*TA*TG and CDRE2 to CAG*TA*AC (modified nucleotides are shown in italics). We then fused these altered promoters to a *lacZ* reporter and monitored their activation upon alkaline stress. As shown in figure 10b, mutation of CDRE1 resulted in a relatively slight decrease in β-galactosidase activity when compared to the wild type. In contrast, modification of CDRE2 causes a much stronger decrease, with a reduction of activity comparable to the absence of calcineurin or Crz1 (figure 10a). This result suggests that CDRE2 is the major Crz1 binding site of *PHO89*.

To test whether Crz1 actually binds to *PHO89* promoter, we used a strain carrying a C-terminally HA-tagged version of Crz1 to perform chromatin immunoprecipitation. As shown in figure 11, after alkaline pH stress Crz1 is recruited very quickly to the region of the promoter that contains the CDRE2 sequence. This recruitment is also transitory: 20 minutes after stress Crz1 is no longer bound to this promoter region. This fast response is in agreement with the fast increase in cytosolic calcium after exposure to alkaline conditions, which peaks at 15-20 seconds after stress (Viladevall *et al.*, 2004). Additionally, a Crz1 version fused with GFP also displays a fast nuclear localization after high pH stress, as previously reported by Ruiz *et al.*, 2008. In summary, the sequential steps needed for the calcineurin-dependent induction of *PHO89* in alkaline conditions (increase in cytosolic calcium from the external medium, activation of the calcineurin, dephosphorylation of Crz1, and entry of Crz1 into the nucleus to bind CDREs) occur in 2 minutes or less after stress.

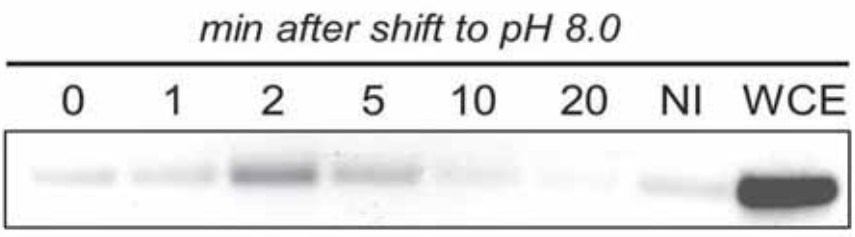

Figure 11. Immunoprecipitated chromatin from SP020 cells (*CRZ1:3xHA*) exposed to pH 8.0 for the indicated times was subjected to PCR amplification for the 143 nt region encompassing the CDRE2 consensus. NI, sample lacking anti-HA antibodies; WCE, whole cell extract.

Crz1 activation during high pH stress would be reinforced by the inhibition of the PKA and Pho80-Pho85 in alkaline conditions (see section 2.4 and 3.3 of Introduction). Both kinases have been described to phosphorylate Crz1 (Kafadar and Cyert, 2004; Sopko *et al.*, 2006), thus their inactivation would promote Crz1 nuclear localization. The strong effect that the calcineurin pathway exerts on *PHO89* would explain why the basal expression of this gene is higher than normal in cells deficient for the protein phosphatase Ppz1, since this mutation results in a constitutively activated calcineurin (Ruiz *et al.*, 2003).

4. The effect of the Rim101 and Snf1 pathways on *PHO89* alkaline induction

Exposure to alkaline conditions triggers the activation of several signaling pathways in *S. cerevisiae*. Apart from the calcineurin and *PHO* pathways, other factors contribute to the adaptive response to this stress by remodeling gene expression and could be possible regulators of *PHO89* alkaline induction. Of those, we were especially interested in Rim101 and Snf1 for two reasons. First, as mentioned in section 1, the promoter of *PHO89* contains putative target sequences for the repressors Nrg1/Nrg2 and Mig1/Mig2, known to be regulated by Rim101 and Snf1 (table 3). Second, the alkaline expression of the Na^+-ATPase *ENA1*, which shares some common traits with *PHO89* (e.g. early response and dependence on calcineurin) is also upregulated by these two pathways (Platara *et al.*, 2006). Moreover, mutant strains lacking Rim101 or Snf1 are sensitive to high pH, suggesting that the response driven by these factors is essential to cope with this stress (Lamb *et al.*, 2001; Platara *et al.*, 2006).

Rim101 is proteolytically activated by high pH stress and was described to govern the expression of several genes under this circumstance (see section 2.1 of Introduction) (Futai *et al.*, 1999; Lamb *et al.*, 2001). Under alkaline conditions, Snf1 becomes activated by Elm1, and also drives the response to this stress by impinging on gene expression (see section 2.3 of Introduction) (Casamayor *et al.*, 2012; Hong and Carlson, 2007).

4.1. Rim101 and Snf1 induce the expression of *PHO89* under alkaline conditions

To explore if the Rim101 and Snf1 pathways have any influence on the induction of *PHO89* after alkaline stress we carried out a β-galactosidase activity assay with strains lacking Rim101 or Snf1 using the plasmid pPHO89-LacZ. As shown in figure 12, the activation of the *PHO89* promoter was reduced nearly at half in a *snf1* strain. Also, the absence of Rim101 caused a similar decrease in the alkaline induction of *PHO89*. Lack of Pho4 had a somewhat stronger impact and the *cnb1* strain displayed the lowest expression. This suggests that, apart from calcineurin and Pho4, both Rim101 and Snf1 have a positive effect on the upregulation of *PHO89* during alkaline pH conditions. It is worth noting that, while this report was in

progress, Pérez-Sampietro and coworkers reported a relationship between Snf1 and *PHO89* expression in the presence of the prooxidant selenite. During stress by selenite, *PHO89* levels increase and this induction depends mainly on Snf1 (Pérez-Sampietro *et al.*, 2013).

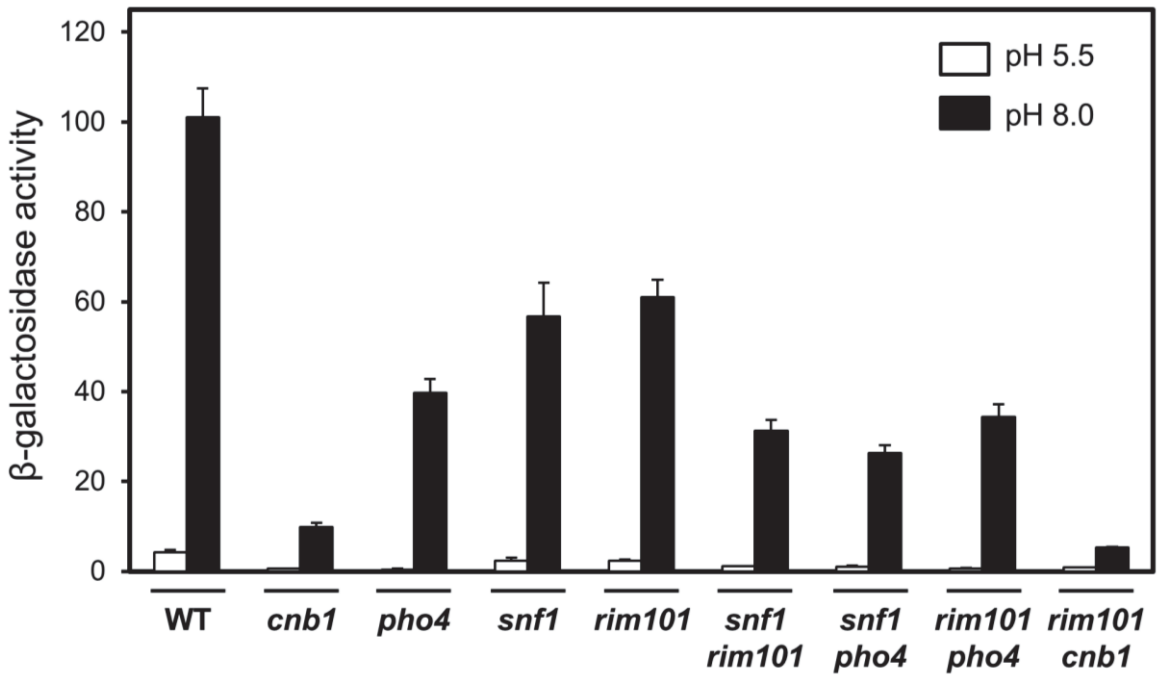

Figure 12. The indicated strains, transformed with reporter pPHO89-LacZ, were exposed to pH 8.0 (closed bars) or maintained at pH 5.5 (empty bars) and β-galactosidase activity determined. Data are mean ± SEM from 10-15 experiments.

In order to determine if any of these pathways shared a common element to induce the expression of *PHO89* we performed the same assay in strains combining some of the mutations already tested (figure 12). It can be observed that deletion of *SNF1* in a strain lacking *rim101* or *pho4* results in a further reduction of promoter induction. Similarly, combining *rim101* and *cnb1* mutations leads to a promoter activity even lower than any of the single mutations, whereas the *rim101 pho4* strain only displays a marginal reduction when compared to the single mutant strains. Although these results suggest that these four proteins control independent signaling pathways acting on the *PHO89* promoter, we cannot discard the possibility that the crosstalk between them could influence the induction of this gene. For example, calcineurin inactivates Elm1, the main Snf1-activating kinase under alkaline conditions, which could negatively affect *PHO89* upregulation (Casamayor *et al.*, 2012; Goldman *et al.*, 2014).

4.2. The absence of Rim101 or Snf1 results in different Pho89 expression kinetics

Next we wanted to confirm the influence of Rim101 and Snf1 on the regulation of *PHO89*. To that end, we decided to investigate the effect of *rim101* and *snf1* mutations in the accumulation of *PHO89* mRNA after alkaline stress. As shown in figure 13a, both strains exhibit reduced levels of *PHO89* when compared to the wild type strain. Interestingly, though, their pattern over time differs considerably. For instance, the *rim101* strain showed a reduced expression mainly at a long-term (30 and 40 minutes), whereas the *snf1* mutation decreased the expression especially at the short-term (5 minutes).

These differences became more evident when checking Pho89 protein accumulation in these strains with a C-terminally HA-tagged version of Pho89. As shown in figure 13b, the short-term response after the alkaline induction (15 and 30 minutes) of the *rim101* strain is essentially identical than that observed in the wild type strain. In contrast, the accumulation of Pho89 at times longer than 30 minutes is drastically diminished. On the other hand, *snf1* mutation caused an overall reduction of Pho89 expression, particularly strong at a short-term, coincident with the peak in Pho89 accumulation observed in the wild type strain.

The differences in the expression of Pho89 over time in these strains may reflect the distinct mechanisms by which Rim101 and Snf1 impinge on its promoter. Thus, we found interesting to explore each of these pathways and their impact on Pho89 alkaline induction in more detail.

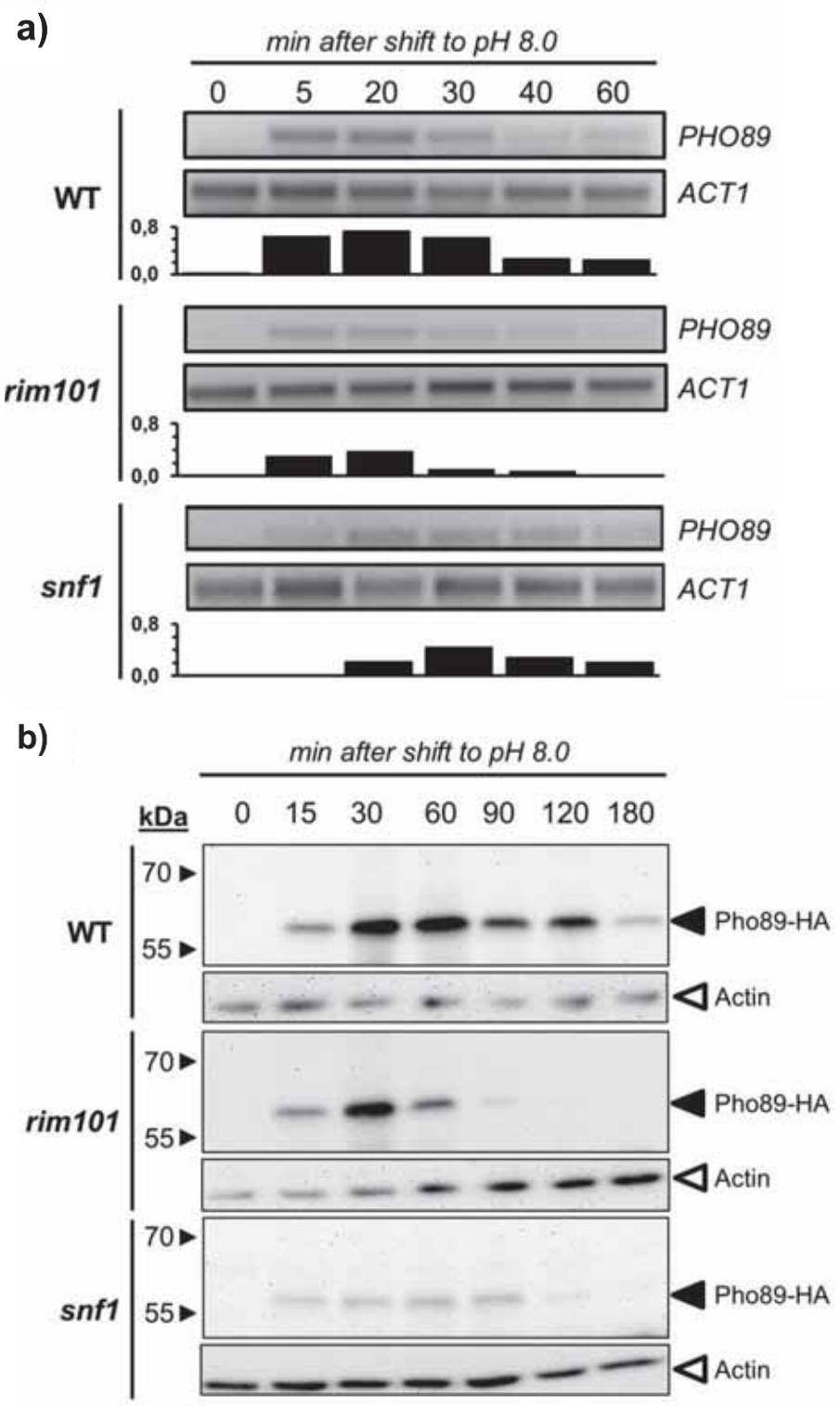

Figure 18. a) Wild type DBY476, RSC21 (*rim101*), and RSC10 (*snf1*) cells were collected at the indicated times after switching the medium to pH 8.0 and total RNA prepared. Semi-quantitative RT-PCR was performed as described in the legend of Figure 7a. Signals were integrated and bars at the bottom of each experiment denote the ratio between the *PHO89* and *ACT1* mRNAs for each time-point and strain. **b)** Strains ASC07 (*PHO89:3xHA*:*HIS3*), ASC11 (*rim101 PHO89:3xHA*:*HIS3*), and ASC09 (*snf1 PHO89:3xHA*:*HIS3*) were subjected to pH 8.0 for the indicated times, processed as in Figure 7b, and the amount of Pho89 revealed by immunoblot.

5. The role of the Rim101 pathway in *PHO89* alkaline induction

Activation of the Rim101 pathway by alkalinization of the medium leads to the activation of Rim101 by proteolysis, enabling its accumulation into the nucleus (Li and Mitchell, 1997). Rim101 acts as a transcriptional repressor: it inhibits the expression of *NRG1*, encoding another repressor (Lamb and Mitchell, 2003); therefore, Rim101 upregulates gene transcription indirectly (see section 2.1 of Introduction).

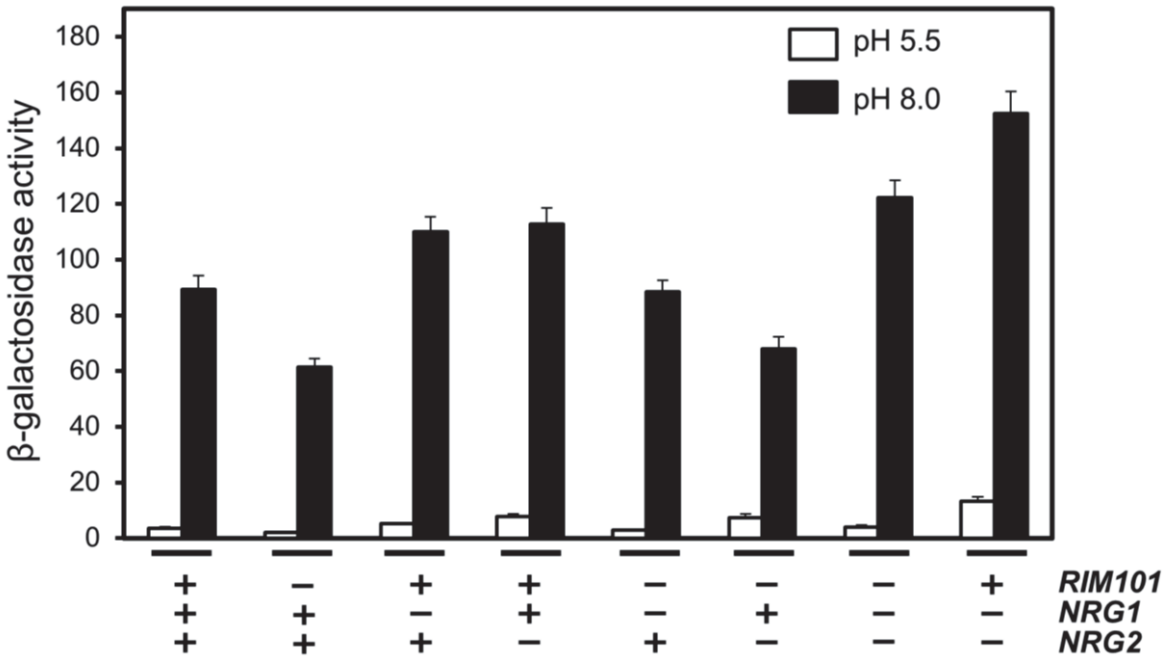

Figure 14. The indicated strains were transformed with plasmid pPHO89-LacZ. Exponential cultures were switched to pH 8.0 (closed bars) and β-galactosidase activity determined after 90 min. Data are mean ± SEM from 20 experiments.

The observation that the absence of Rim101 impaired *PHO89* alkaline induction (figure 13) and the presence of putative Nrg1/Nrg2 binding sites on the transporter gene promoter (table 3) prompted us to investigate if Rim101 was controlling *PHO89* expression through these repressors. To this end, we evaluated the induction of the reporter plasmid pPHO89-LacZ after high pH stress in strains carrying the *rim101*, *nrg1*, or *nrg2* mutation and their combinations. As shown in figure 14, *nrg1* and *nrg2* strains display increased levels of β-galactosidase activity compared to the wild type strain, suggesting that both repressors exert a negative effect on the promoter of *PHO89*. In fact, the absence of both Nrg1 and Nrg2 gives rise to a higher induction than any of the single mutations does. However, the response in a

rim101 nrg1 background was similar to that of the wild type strain, whereas the expression level in a *rim101 nrg2* strain was only slightly higher than that of the *rim101* strain. This may reflect that, in response to alkaline pH, Rim101 impinges on *PHO89* preferentially through the repression of Nrg1. It seems that, apart from the Nrg repressors, Rim101 is upregulating the *PHO89* promoter through another mechanism, since deleting Rim101 in an *nrg1 nrg2* background causes a decrease in the activation of pPHO89-LacZ.

Nrg1 and Nrg2 possess highly similar DNA-binding domains and regulate a similar set of genes in response to carbon stress or several environmental stresses, such as osmotic, salt, or alkaline pH stresses (Vyas *et al.*, 2001, 2005). However, their own regulation differs considerably. While *NRG1* is induced under acidic conditions and repressed under high pH by Rim101 (Causton *et al.*, 2001; Lamb and Mitchell, 2003), *NRG2* is slightly upregulated by alkaline stress (Lamb *et al.*, 2001). Moreover, during carbon stress, Nrg1 protein levels increase but Nrg2 levels decrease due to a posttranscriptional effect (Berkey *et al.*, 2004). In summary, even though Nrg1 and Nrg2 share some common targets, they might be repressing gene transcription under different circumstances or at different periods during the adaptation to specific stress. In the case of *PHO89*, our results suggest that its induction under alkaline conditions by the Rim101 pathway relies mainly on *NRG1* repression. Nrg2 also seems to repress *PHO89* but its regulation by Rim101 is less clear.

These results are important to understand the kinetics of Pho89 accumulation observed in the *rim101* strain in figure 13. Activating *PHO89* expression by inhibiting the transcription of its repressor would take a longer time than, for example, binding of Crz1 to *PHO89* promoter. Even if Rim101 could repress *NRG1* transcription rapidly after alkaline pH stress, the actual Nrg1 protein already present would need to be degraded for the derepression to take effect. Thus, it is conceivable that *rim101* cells exhibit wild type levels of Pho89 at a short-term after the stress, but show a decreased accumulation of this transporter at longer time points, when the inability to alleviate Nrg1-mediated repression becomes patent.

6. The role of the Snf1 pathway in *PHO89* alkaline induction

The SNF1 kinase complex regulates several cellular processes in response to carbon stress i.e. glucose scarcity or growth on non-fermentable carbon sources. Under this situation, Snf1 has been described to govern gene transcription, protein stability, and enzyme activity. This kinase contributes to the remodeling of gene expression by controlling the activity of several transcription factors, such as Cat8, Sip4, or the repressors Mig1/Mig2 and Nrg1/Nrg2. In general, this leads to the upregulation of genes related to gluconeogenesis and the utilization of carbon sources other than glucose (see section 2.3 of Introduction). Alkalinization of the medium also results in the phosphorylation of Snf1 by the upstream kinase Elm1 and its translocation into the nucleus (Casamayor *et al.*, 2012; Hong and Carlson, 2007). Snf1 upregulates 38% of the short-term induced genes in alkaline conditions, most of them related to the response to low glucose conditions (Casamayor *et al.*, 2012). Our observation that *snf1* mutation leads to a decreased expression of *PHO89* (figure 13) encouraged us to investigate the mechanism by which this regulation takes place.

6.1. Impact of the Snf1 pathway on *PHO89* through Nrg1 and Nrg2

In conditions of glucose scarcity, the repressor function of Nrg1 and Nrg2 was found to be inhibited by Snf1, thus relieving glucose-driven repression (Kuchin *et al.*, 2002). It was reported that Snf1 interacts with both repressors *in vitro*, although their phosphorylation could not be detected (Vyas *et al.*, 2001). Having observed that Nrg1 and Nrg2 act negatively on *PHO89* expression, we decided to test if Snf1 was involved in the derepression of *PHO89* under alkaline conditions by regulating Nrg1 and/or Nrg2.

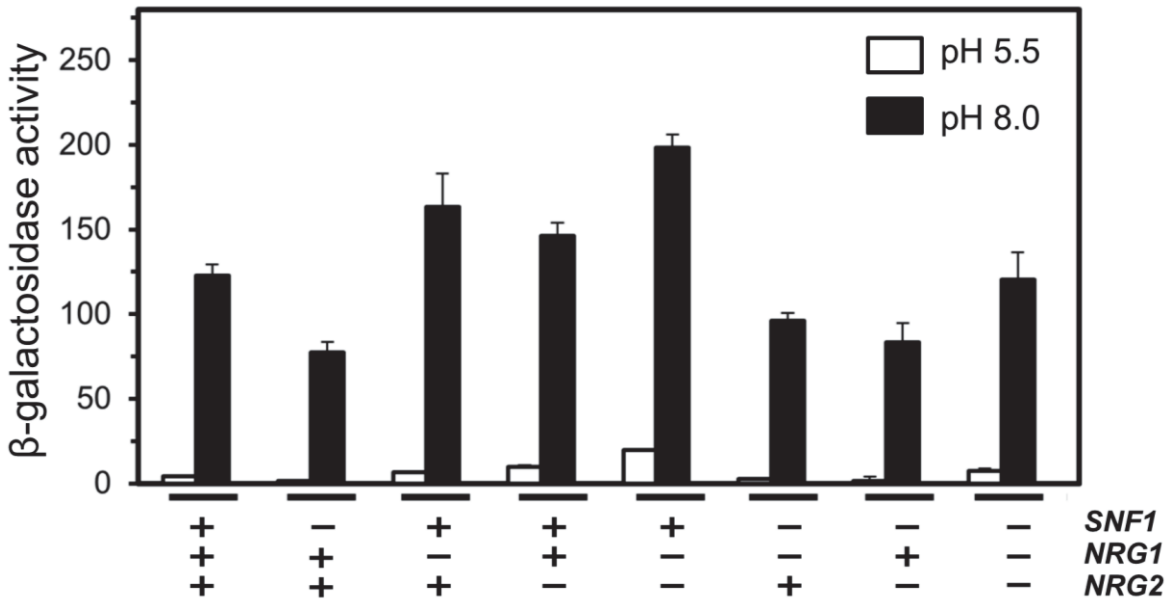

Figure 15. The indicated strains were transformed with plasmid pPHO89-LacZ. Exponential cultures were switched to pH 8.0 (closed bars) and β-galactosidase activity determined after 90 min. Data are mean ± SEM from 12-15 experiments.

To that end, we monitored the expression of β-galactosidase under the control of the *PHO89* promoter in wild type, *snf1*, *nrg1*, and *nrg2* strains, and their combinations. As shown in figure 15, combining the *snf1* mutation with either *nrg1* or *nrg2* mutations results in a response that is intermediate between the observed for *snf1* and wild type strains. Furthermore, the strain *snf1 nrg1 nrg2* exhibits the same promoter activity than the wild type strain, and lower than the *nrg1 nrg2* strain. These results, without discarding a possible inhibition of Nrg1/Nrg2 by Snf1, suggest the existence of additional regulatory elements under the control of Snf1 impinging on *PHO89* alkaline induction.

6.2. Snf1 upregulates *PHO89* through Mig2

The fact that Snf1 has a positive effect on the induction of *PHO89* after high pH stress and the presence of a putative Mig1/Mig2 binding site on the *PHO89* promoter prompted us to speculate if the Snf1 kinase would inhibit Mig1 or Mig2 under alkaline conditions, thus derepressing *PHO89*. To confirm this hypothesis we performed a β-galactosidase reporter assay with the plasmid pPHO89-LacZ employing the strains *snf1*, *mig1*, *mig2*, and all their possible combinations (figure 16). Curiously, some interesting results can be observed even in non-stressed cells (pH 5.5). For instance, the absence of Mig1 leads to similar levels of β-galactosidase activity than that observed in the wild type (1.73 ± 0.23 and 1.41 ± 0.21 units,

respectively); in contrast, the *mig2* strain consistently shows higher levels (4.51 ± 0.29 units). The prominent role Mig2 in repression of the *PHO89* promoter can also be observed in alkaline induced cells: while the *mig1* mutation does not affect the expression of the reporter, lack of Mig2 results in higher levels of activity than the wild type. Furthermore, the *snf1 mig1* strain displays the same reduced levels of induction than the single *snf1* mutant strain, indicating that deleting Mig1 in a strain without Snf1 activity does not restore normal *PHO89* induction. On the other hand, the mutation of Mig2 in the *snf1* strain resulted in an induction higher than that of the wild type strain and comparable to the *mig2* strain. These results suggest that Mig2, but not Mig1, represses the *PHO89* promoter and that an alkaline pH-activated Snf1 might relieve this repression.

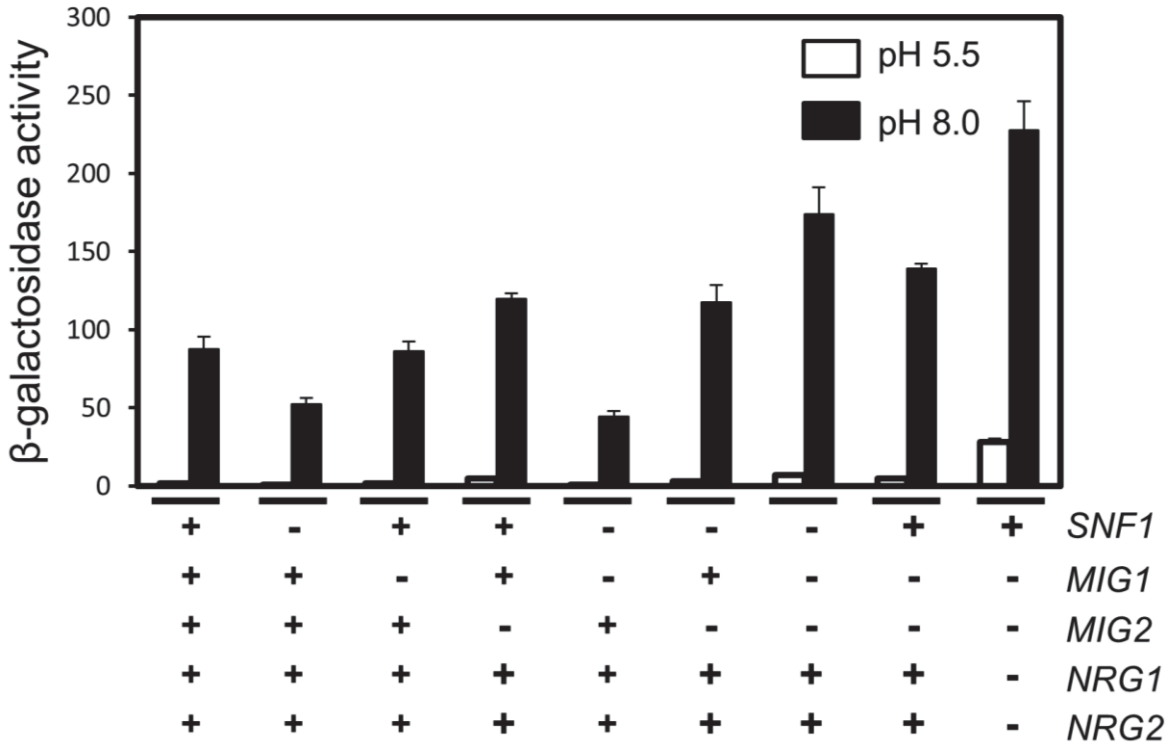

Figure 16. The indicated strains were transformed with plasmid pPHO89-LacZ. Exponential cultures were switched to pH 8.0 (closed bars) and β-galactosidase activity determined after 90 min. Data are mean ± SEM from 9-12 experiments.

Interestingly, the absence of both Mig1 and Mig2 showed slightly augmented promoter activity when compared to the *mig2* strain, suggesting that, in cells lacking Mig2, Mig1 might exert some repression on *PHO89*. These results were compatible with the possibility that, under alkaline conditions, Mig1 and Mig2 could be differently regulated by

Snf1; thereby, it could be that only one of them would mediate the Snf1 input on *PHO89* during this stress.

The observation that, in the absence of *MIG1* and *MIG2*, further deletion of *NRG1* an *NRG2* results in an even higher promoter activity suggests that these two groups of repressors act independently on *PHO89*. Remarkably, we observed an unexpectedly higher induction in the *snf1 mig1 mig2* strain when compared to the *mig1 mig2* double mutant. This might be due to additional regulation mechanisms controlled by Snf1, since this kinase have been described to affect gene transcription at multiple levels (see section 2.3 of Introduction).

6.3. Mig1 and Mig2 are transiently phosphorylated by alkaline stress in a Snf1-dependent manner

Under glucose starvation conditions, Snf1 negatively regulates Mig1 repression function by phosphorylation (Ostling and Ronne, 1998; Treitel *et al.*, 1998). In view of the lesser role that Mig1 seems to play in *PHO89* alkaline induction, we wondered if this repressor is actually regulated by phosphorylation after high pH stress. To that end, we generated a C-terminally HA-tagged version of the repressor in order to determine its phosphorylation state, taking advantage of the fact that a phosphorylated Mig1 displays a mobility shift in a SDS-PAGE.

As shown in figure 17a, exposure to alkaline pH results in the appearance of slower migrating bands shortly after (2 min) the onset of stress. This effect is transient since 10 min after the induction the Mig1 signal returned to its initial mobility. To determine if the mobility shift was due to phosphorylation, prior to the SDS-PAGE all samples were treated with alkaline phosphatase with or without EDTA, which blocks the phosphatase activity. As it can be observed, the samples without EDTA show no mobility shift at all after alkaline stress, indicating that the slower migration corresponds to phosphorylated versions of Mig1. To assess the role of Snf1 in this phosphorylation, we then performed the same experiment using a *snf1* strain. In the absence of Snf1, Mig1 does not display any mobility shift, indicating that its phosphorylation after high pH stress is dependent on Snf1.

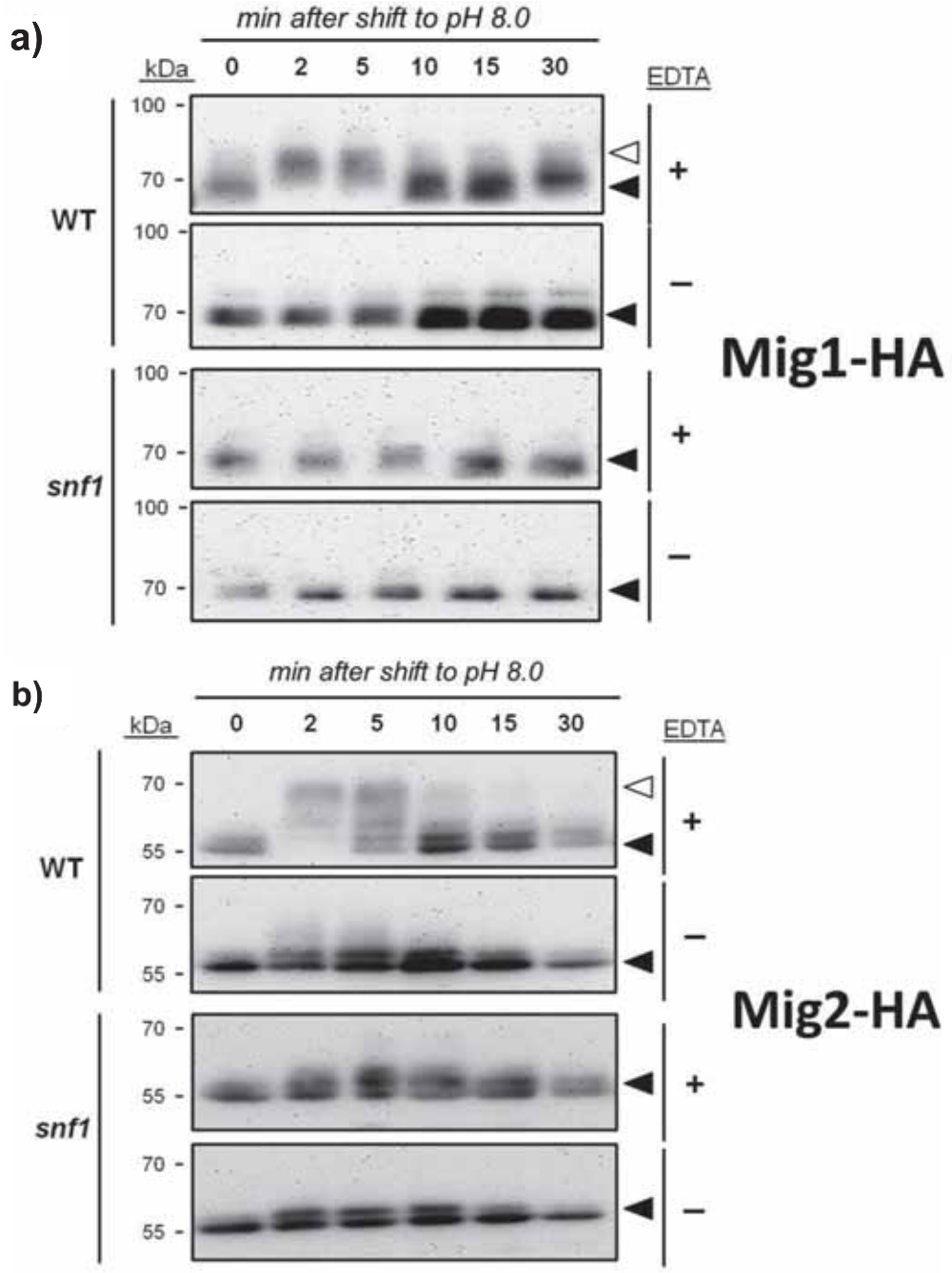

Figure 17. a) A chromosomal copy of Mig1 including a C-terminal 3xHA epitope tag was introduced in wild type BY4741 cells and in its *snf1* derivative. After exposing cells to pH 8.0 for the indicated times, extracts were prepared and subjected to SDS-PAGE (10% polyacrylamide gels), prior treatment with alkaline phosphatase in the absence (-) or the presence (+, to prevent the action of the phosphatase) of 50 mM EDTA. Immunoblots were performed using anti-HA antibodies. Empty triangles denote slower (more phosphorylated) species. **b)** A chromosomal copy of Mig2 including a C-terminal 3xHA epitope tag was introduced in wild type BY4741 cells and in its *snf1* derivative. Cells and extracts were treated as described above.

Interestingly, Snf1 has been reported to accumulate into the nucleus during carbon stress but not during salt stress, even though it becomes activated in both situations (Hong and Carlson, 2007; Vincent *et al.*, 2001). This correlates well with Snf1-dependent Mig1

phosphorylation: glucose scarcity causes phosphorylation of Mig1 whereas salt stress does not (McCartney and Schmidt, 2001; Treitel *et al.*, 1998). Because the exposure to alkaline pH translocates Snf1 into the nucleus, as it happens for carbon stress, it was not surprising to find that Mig1 becomes phosphorylated in a Snf1-dependent manner in alkaline conditions.

As shown in figure 16, Mig2 plays a major role in *PHO89* repression when compared to Mig1. Therefore, we wanted to determine if this repressor was also regulated by phosphorylation in alkaline conditions. The output of this experiment was not easy to predict because it has been accepted for many years that Snf1 regulates by phosphorylation Mig1 but not Mig2 (Lutfiyya *et al.*, 1998). As it can be observed in figure 17b, Mig2 shows a mobility profile very similar to that of Mig1: it migrates slowly 2 min after high pH stress and returns to its initial mobility 10 min after the induction. This shift was abolished in samples treated with alkaline phosphatase without EDTA, indicating that it was caused by phosphorylation of Mig2. Alike the case of Mig1, this phosphorylation was dependent on Snf1, since in a *snf1* strain Mig2 does not show slower migrating bands in response to high pH stress.

In spite of the failure to associate Snf1 with the phosphorylation of Mig2, a recent report described that shifting cells from a glucose- to a galactose-containing medium induced a fast degradation of Mig2 by a mechanism that involves Snf1-dependent Mig2 phosphorylation (Lim *et al.*, 2011). Curiously, the authors reported an almost complete depletion of Mig2 after only 20 min upon shifting cells to galactose, whereas in our experiment the amount of Mig2 did not drastically change after 30 min of alkaline stress. This suggests that the fate of Mig2 after being phosphorylated may differ between both stresses. In any case, the evidence supporting Mig2 phosphorylation in response to stress is worth to highlight since, in contrast to Mig1, this post-translational modification has not been captured by the diverse phospho-proteomics approaches reported so far.

6.4. Mig1 and Mig2 exit the nucleus during high pH stress

After carbon stress, Snf1 derepresses gene transcription by phosphorylating Mig1, which causes the dissociation of this repressor with the co-repressor complex Ssn6-Tup1 and promotes its interaction with the exportin Msn5, resulting in its nuclear exclusion (DeVit and

Johnston, 1999; Papamichos-Chronakis *et al.*, 2004). Having observed that both Mig1 and Mig2 become phosphorylated upon alkaline stress in a Snf1-dependent fashion, we wondered if this posttranslational modification corresponded with a change in their subcellular localization. To that end, we monitored Mig1 and Mig2 localization using chromosomally integrated constructs expressing a GFP-fused version of these repressors. In the case of Mig2-GFP, the strain BY4741 carrying the chromosomal integration provided an insufficient fluorescent signal. Thus, we constructed the same fusion in the DBY746 background, which yielded a better signal.

As shown in figure 18a, in non-stressed cells Mig1 was localized mostly in the nucleus. In contrast, exposure to alkaline pH stress triggered a fast (3 min) cytosolic distribution of the repressor. This shift was transient, since only after 10 min of stress Mig1 was again concentrated in the nucleus. The same experiment using a GFP-tagged Mig2 showed very similar results: Mig2 displays a nuclear localization at acidic pH and translocates from the nucleus to the cytoplasm in 3 min after high pH stress, relocating into the nucleus in 10-15 min (figure 18b). Although Mig2 was believed to remain nuclear after carbon stress (Lutfiyya *et al.*, 1998), a recent report, using a chromosomally GFP-tagged version of the repressor instead of expressing it from a plasmid, described changes in the localization of this protein (Fernández-Cid *et al.*, 2012). The authors showed that, upon shift to low-glucose conditions or after oxidative stress, Mig2 translocates from the nucleus to the mitochondria, where it is involved in mitochondria fusion. Whereas in this work Fernández-Cid and coworkers did not explore the dependence on Snf1 for Mig2 translocation, in our case, we did not observe any mitochondrial localization of Mig2.

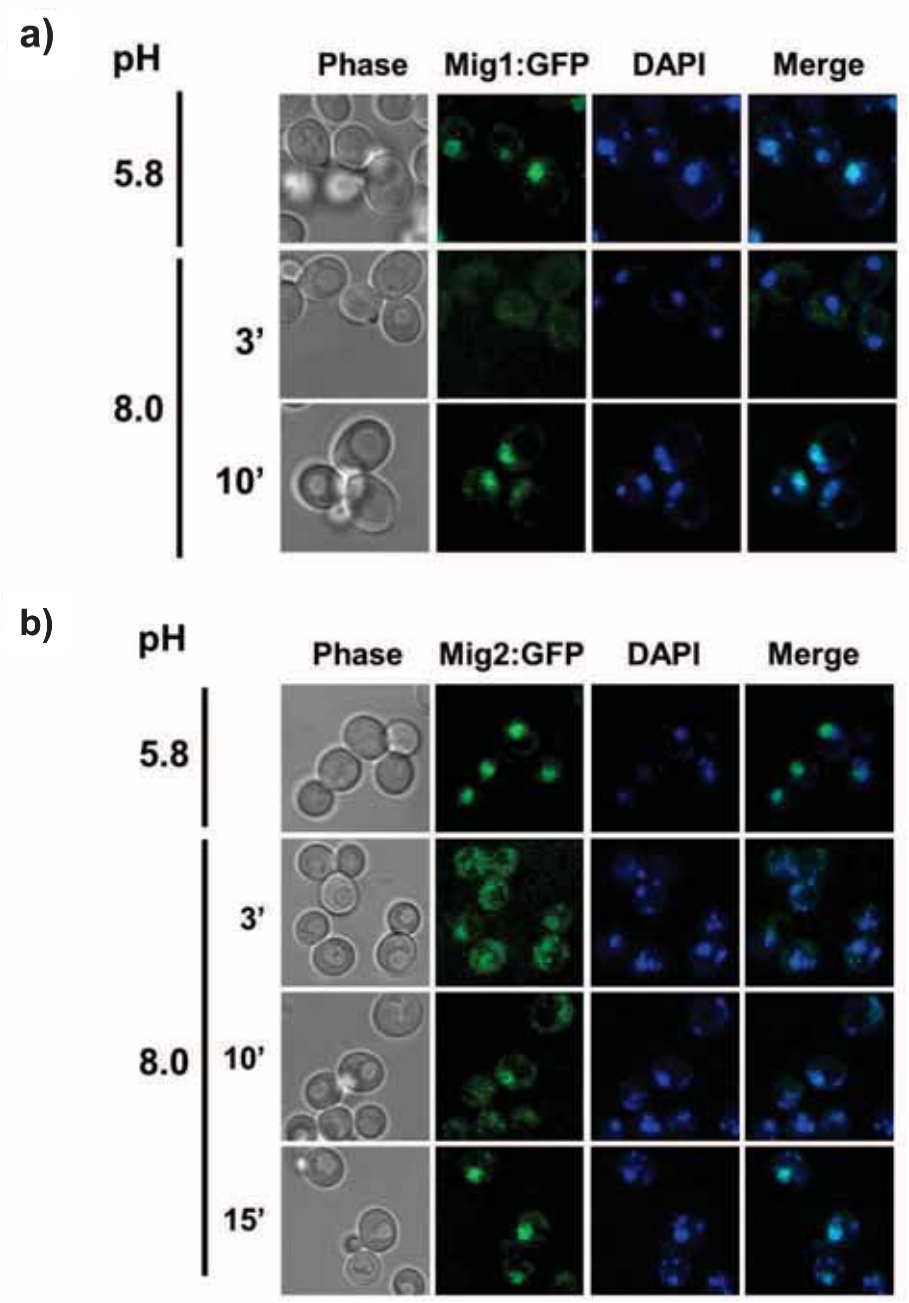

Figure 18. Strains SP018 (a) and SP048 (b), containing chromosomally encoded C-terminal fusions of GFP with Mig1 and Mig2 respectively, were shifted to pH 8.0 and the localization of the repressor followed by fluorescence confocal microscopy. Nuclei were stained with DAPI to illustrate nuclear co-localization of the GFP and DAPI signals (merged).

In summary, alkaline pH stress promotes a fast but brief Snf1-mediated phosphorylation of Mig1 and Mig2, which correlates with their shift from the nucleus to the cytoplasm. This suggests that the preeminent role of Mig2 over Mig1 in *PHO89* repression is not due to a differential regulation of these repressors in response to alkaline conditions but most likely to a distinct affinity for the target sequence found on the promoter of *PHO89*. This would explain why the repressor activity of Mig1 on *PHO89* only takes place in the

absence of Mig2 (figure 16). The opposite case has been described for the glucose-repressed genes *SUC2* and *DOG2*: they are clearly repressed by Mig1 and repression by Mig2 only becomes apparent in the absence of Mig1 (Lutfiyya *et al.*, 1998). The authors speculated that although Mig1 and Mig2 have the same DNA-binding site, thus recognizing the same DNA sequence, their affinities for binding to it differ, hence explaining why in some promoters Mig1 and Mig2 contribute differently to its repression (Lutfiyya and Johnston, 1996; Lutfiyya *et al.*, 1998). It is worth mentioning that *PHO89* was reported to be coregulated by Mig1 and Mig2 in Westholm *et al.*, 2008. The authors showed transcriptomic data, based on microarray experiments at standard growth conditions, in which *PHO89* expression did not show a significant increase in a *mig1* strain when compared to the wild type (1.3-fold) but the *mig1 mig2* strain displayed a 56-fold increase. In our opinion, these results point more toward a predominant role of Mig2 rather than a strict coregulation, in agreement with our own results.

6.5. Snf1 is rapidly but transiently phosphorylated after alkaline pH stress

Snf1 catalytic activity requires the phosphorylation of its Thr210; thus, the phosphorylation state of this residue is often used as an indicator of Snf1 activity. It was previously reported that alkalinization of the medium results in Snf1 Thr210 phosphorylation within 5 min and that 1 hour after the stress it was no longer phosphorylated (Hong and Carlson, 2007). Our previous results show that Mig1 and Mig2 are phosphorylated 2 min after high pH stress and that this modification depends on the presence of Snf1 (figure 17). If the catalytic activity of Snf1 is also required for the phosphorylation of these repressors in alkaline conditions, Snf1 must be phosphorylated prior to Mig1 and Mig2. To determine this, we monitored Snf1 Thr210 phosphorylation in a time course after alkaline pH stress (figure 19). It can be observed that Snf1 phosphorylation is almost immediate (30 sec) after high pH stress, but also very transient, since at 10-15 min after the induction it has returned to basal levels.

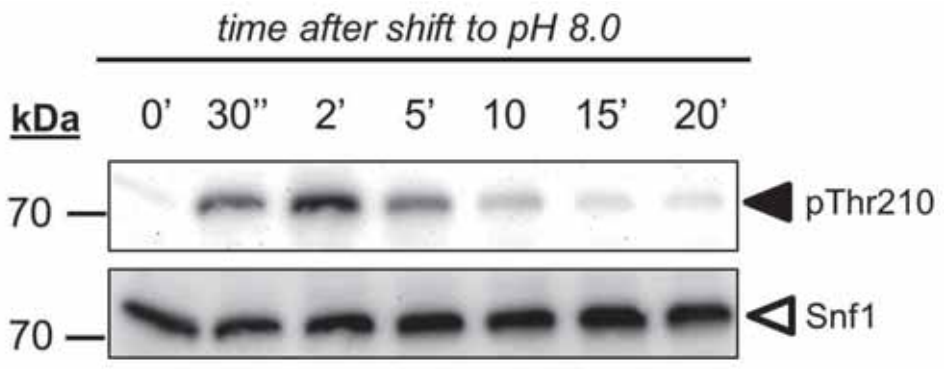

Figure 19. Strain DBY746 was subjected to alkaline shift for the indicated periods and samples (10 µl) processed for SDS-PAGE (10% gels) and immunoblot was performed using anti-phospho-Thr172-AMPK (phosphorylated Snf1, pThr210) or anti-His antibodies (Snf1 protein, Snf1).

This kinetics correlate perfectly with those observed after alkaline stress in figures 17 and 18: Mig1 and Mig2 phosphorylation and translocation occur very fast (2-3 min) and return back to their initial state shortly after (10-15 min), suggesting that the Snf1 catalytic activity governs this processes. This would mean that the alkaline upregulation of *PHO89* by the Snf1 pathway, through Mig2-mediated derepression, occurs shortly after stress and lasts only for a short time. In fact, as reported in figure 13b, the *snf1* strain shows a strong decrease of Pho89 protein levels already at the short-term.

6.6. The absence of Reg1 causes a drastic upregulation of Pho89

The Glc7-Reg1 protein phosphatase is the main element that dephosphorylates Snf1, thus inactivating the kinase (see section 2.3 of Introduction). Therefore, a strain lacking Reg1 possesses constitutively active Snf1 (Hong *et al.*, 2005). To further understand the impact of the Snf1 pathway in *PHO89* alkaline induction, we decided to investigate the effects of a *reg1* mutation in the expression of this transporter.

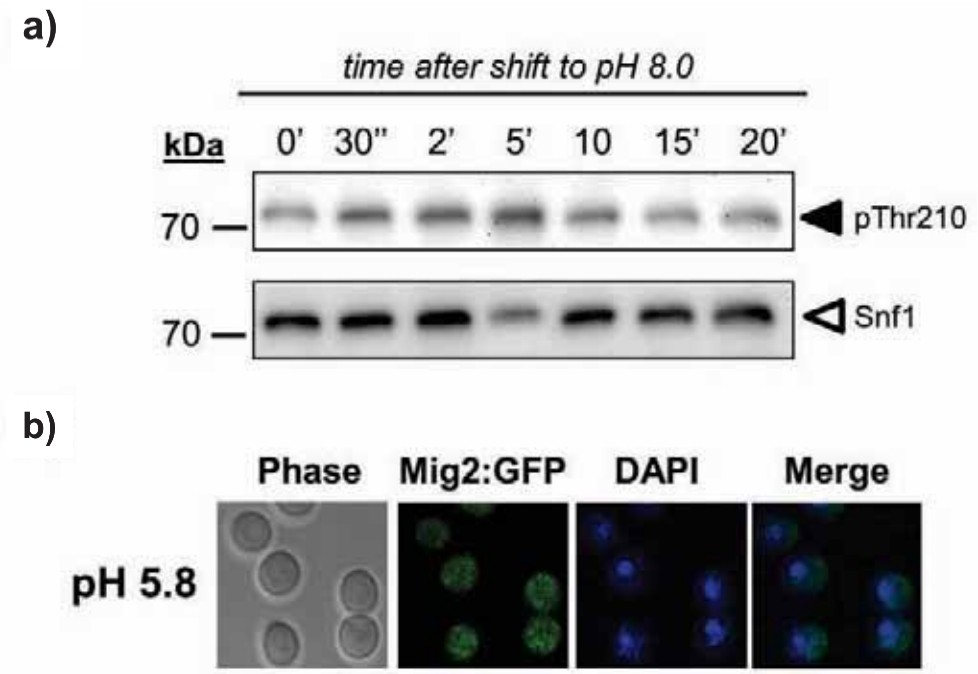

Figure 20. a) Strain RSC89 (*reg1*) was subjected to alkaline shift for the indicated periods and samples were subjected to immunobloting for phosphorylated Snf1 as indicated in figure 19. **b)** The localization of Mig2 was followed in strain ASC34 (*reg1 MIG2-GFP*). Nuclei were stained with DAPI to illustrate nuclear co-localization of the GFP and DAPI signals (merged).

First, we performed an immunoblot to determine the phosphorylation state of Snf1 in a *reg1* strain following the same conditions as in figure 19. As shown in figure 20a, the absence of Reg1 causes phosphorylation of Snf1 even in non-stressing conditions (pH 5.5). Moreover, phosphorylation was further increased by high pH stress. Interestingly, these basal levels of phosphorylated Snf1 are in agreement with the localization of Mig2, which is excluded from the nucleus at pH 5.8 (figure 20b).

We then speculated that, if *reg1* cells show a constitutive Mig2 nuclear exclusion, *PHO89* would become permanently derepressed by this transcription factor. To examine if this could have an impact on the *PHO89* promoter, we carried out a β-galactosidase activity assay in strains wild type, *reg1*, and *reg1 snf1*. As shown in figure 21a, the absence of Reg1 resulted in a drastic upregulation of the *PHO89* promoter activity when compared to the wild type, in both non-induced and stressed cells. Interestingly, further deletion of *SNF1* in the *reg1* strain not only abolished the dramatic induction found in the *reg1* mutant, but brought down the activity of the promoter to levels comparable to those found for the single mutant strain *snf1* (figures 12, 15 and 16), confirming that the effects observed in a *reg1* strain are due to a hyperactive Snf1.

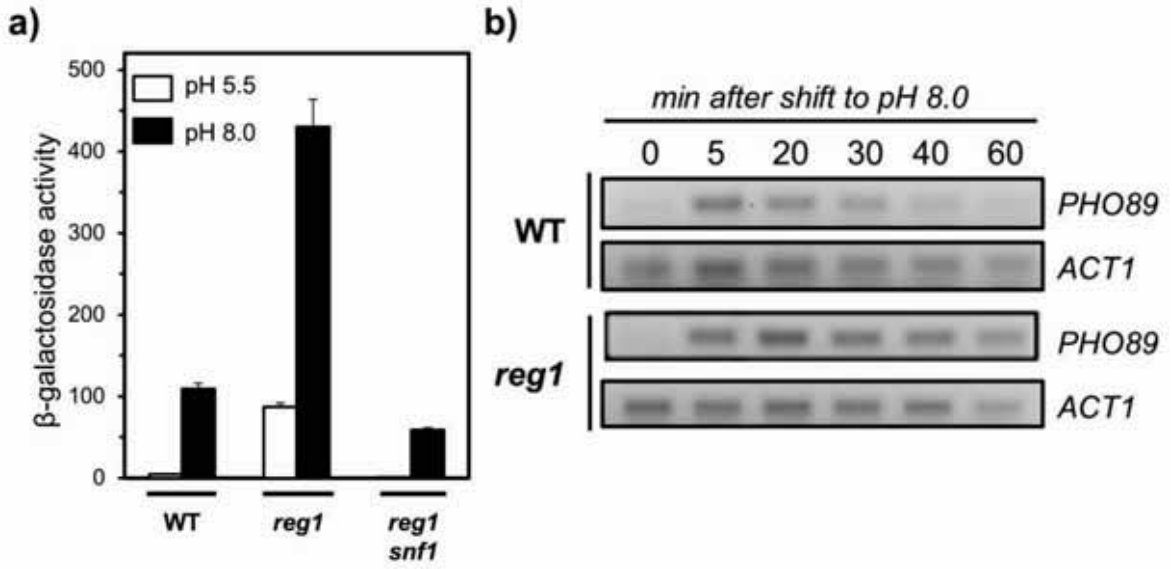

Figure 21. **a)** Wild type strain DBY746 and its *reg1* and *reg1 snf1* derivatives were transformed with pPHO89-LacZ and subjected to high pH stress (pH 8.0, closed bars) prior determination of β-galactosidase activity. **b)** Wild type DBY746 and RSC89 (*reg1*) cells were collected at the indicated times after switching the medium to pH 8.0 and total RNA prepared. Semi-quantitative RT-PCR was performed as described in the legend of Figure 7a.

To determine the *PHO89* mRNA levels in this situation, we performed a RT-PCR experiment comparing the wild type and *reg1* strains in a time course after high pH stress (figure 21b). In contrast to what we observed in the β-galactosidase activity assay, in this experiment *reg1* cells did not display significantly increased levels of *PHO89* in basal conditions. In fact, the main difference between both strains occurs at a long-term, when the *reg1* strain shows a higher persistence of *PHO89* mRNA. At 40 or 60 min after stress, the wild type strain exhibits already reduced levels of *PHO89* whereas the *reg1* mutant strain still displays a significant expression. The results obtained so far provide a possible explanation to this observation. According to what we observed for the kinetics of Crz1 and Mig2, the positive signals impinging on *PHO89* right after alkaline stress (induction by Crz1 and derepression by Mig2) would only last for 15 min approximately, which would be the reason why *PHO89* mRNA levels start decreasing at 20 minutes after the induction (figure 7a). In contrast, a strain lacking Reg1 would not repress back *PHO89* through Mig2, resulting in a longer induction of *PHO89* after high pH stress.

Next, we wanted to investigate the effects of the *reg1* mutation on Pho89 protein levels. To that end, we introduced a chromosomally HA-tagged version of Pho89 into the mutant strains of interest and performed immunoblot experiments. As shown in figure 22, the wild type and *reg1* strains display a similar Pho89 accumulation at a short-term (15 and 30 min). In contrast, while in the wild type strain Pho89 levels decrease from 30 min onward, the *reg1* strain shows a much stronger persistence of this protein after high pH stress.

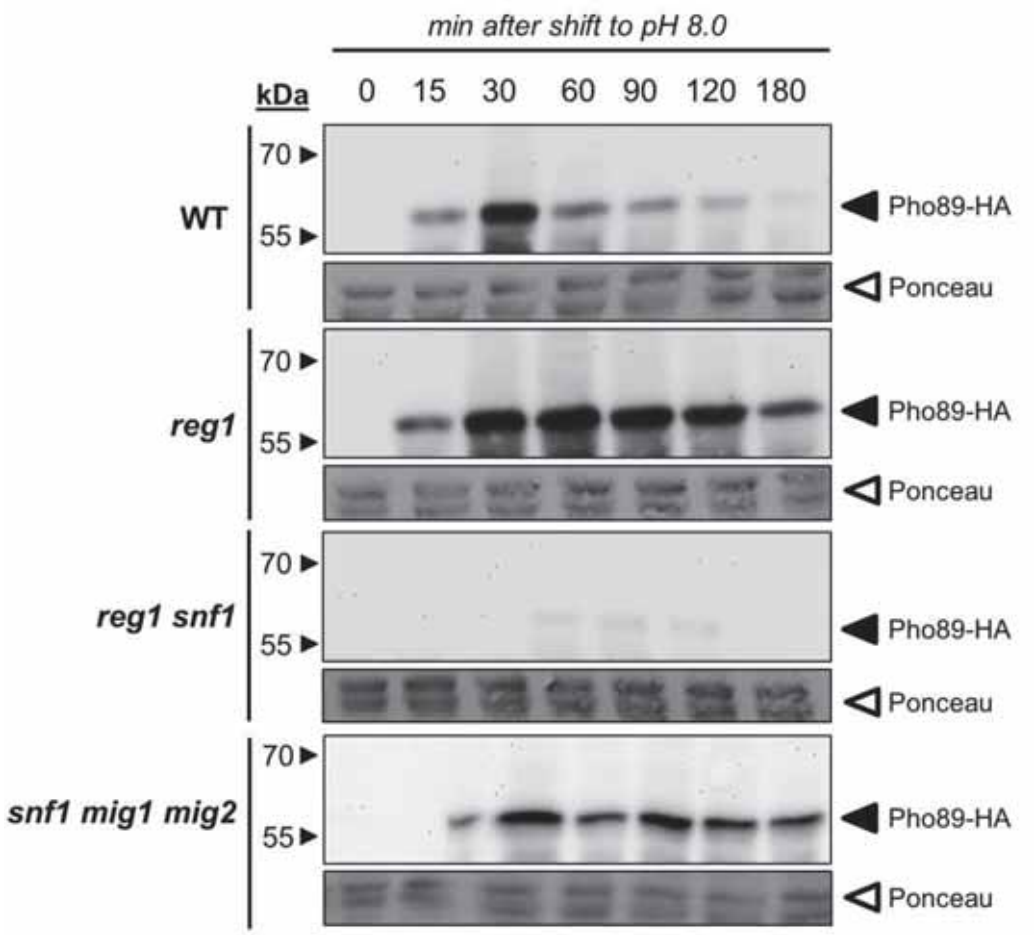

Figure 22. Cultures of strains ASC07 (*PHO89:3xHA:HIS3*), ASC24 (*reg1 PHO89:3xHA:HIS3*), ASC25 (*reg1 snf1 PHO89:3xHA:HIS3*), and ASC45 (*snf1 mig1 mig2 PHO89:3xHA:HIS3*) were shifted to pH 8.0 and protein extracts were prepared. Samples (30 μg of protein) were resolved by SDS-PAGE and processed for immunoblot as described in the legend of Figure 7b.

The addition of the *snf1* mutation to the *reg1* strain resulted in a reduction of the Pho89 protein levels comparable to those observed in the *snf1* single mutant (figure 13b). This indicates that the Pho89 upregulation caused by the lack of Reg1 is fully due to its effect on Snf1 and not to other mechanisms, in agreement with the results shown in figure 21. Additionally, the Pho89 expression profile in a *snf1 mig1 mig2* strain resembles that of the

reg1 mutant strain. This would indicate that most of the positive effect that the Snf1 pathway exerts on Pho89 alkaline induction is mediated through the inhibition of Mig1 and Mig2.

In summary, our results demonstrate that alkaline stress triggers a rapid phosphorylation of Snf1, which probably phosphorylates the repressors Mig1 and Mig2 leading to their nuclear exclusion. However, this activity only lasts for 15 min approximately; after that, Snf1 is dephosphorylated, probably as a preamble of the dephosphorylation of Mig1 and Mig2, which translocate back to the nucleus. In terms of *PHO89* induction, this would mean that the relief of Mig2 repression would only last for 15 min, since this repressor is back into the nucleus at that time. Therefore, a strain with a constitutively inactive Mig2 (i.e. a *reg1* strain) might not display high levels of *PHO89* at basal conditions, since in this circumstance it lacks the positive input of Crz1 and Pho4. On the other hand, after high pH stress, this strain would be unable to repress *PHO89* back to its basal levels, accounting for a longer expression of this transporter. Surprisingly, in the experiment using a β-galactosidase reporter the basal activity of the *PHO89* promoter in a *reg1* strain was nearly as high as the wild type under alkaline conditions (figure 21a). In contrast, the *reg1* strain does not show any significant increase in the levels of *PHO89* mRNA and protein at acidic pH (figures 21b and 22). It must be noted that *lacZ* is expressed under the control of the *PHO89* promoter but it does not share the elements of *PHO89* that might determine the stability of its mRNA, such as the 3' UTR. Similarly, β-galactosidase is a very stable protein, which apparently is not the case for Pho89. Therefore, these differences could be attributed to a higher stability of the *lacZ* mRNA or protein: in a *reg1* strain at basal conditions, the derepression by Mig2 of the *PHO89* promoter could be enough to accumulate significant levels of β-galactosidase but not of *PHO89* mRNA or protein.

Nevertheless, the increased amounts of Pho89 at 120 or 180 minutes in the *reg1* strain may not be only due to Mig2 inability to repress *PHO89*. It was recently reported that the lactate transporter Jen1 is regulated by Glc7-Reg1 and Snf1 at both the mRNA and protein levels. In cells growing with lactate as carbon source, *JEN1* is induced in a Snf1-dependent manner (Bojunga and Entian, 1999). In addition, Snf1 phosphorylates and inhibits the arrestin-like protein Rod1, responsible for Jen1 degradation, thus increasing Jen1 stability (Becuwe *et al.*, 2012). When cells are shifted to a glucose-containing medium, Glc7-Reg1 dephosphorylates Rod1, promoting Jen1 ubiquitination and degradation. Moreover, the

endocytosis of the low-affinity glucose transporters Hxt1 and Hxt3 is also downregulated by Snf1 through Rod1 and its paralog Rog3 (O'Donnell *et al.*, 2014). Therefore, it is possible that, in part, the Pho89 increased levels observed in *reg1* cells would be the result of an inhibition of Pho89 degradation mediated by an hyperactive Snf1, thus extending the transporter half-life. On the other hand, the *snf1* strain would lack a mechanism to prevent Pho89 degradation, leading to a decreased accumulation of this protein. This would account for some of the differences observed between the wild type and the *snf1* strain. For instance, as shown in figure 13a, *snf1* cells display wild type levels of *PHO89* mRNA at a long-term (40 minutes); however, the protein levels in this same strain at 90 or 120 minutes after the alkaline stress are considerably lower than the wild type's. It is worth mentioning that the internalization of the pheromone receptor Ste2 requires dephosphorylation of Rod1 mediated by calcineurin (Alvaro *et al.*, 2014). Taking all this into account, some of the pathways described in this work to drive *PHO89* induction under alkaline conditions may also exert another layer of regulation at the posttranslational level.

In summary, we report that the induction under alkaline conditions of *PHO89* depends on the activation of four different pathways: the *PHO* pathway, the calcineurin pathway, the Rim101 pathway, and the Snf1 pathway. This complex regulatory network would be responsible for the differences between *PHO84* and *PHO89* expression after high pH stress not only in the kinetics of the response, but also in the sensitivity to changes in pH. In this regard, the *PHO84* promoter was described to respond to slight increases in the pH of the medium and to display a near maximal response at pH 7, while the *PHO89* promoter was practically non-induced at pH 7 and its maximal response corresponded to pH 8.2 (Serrano *et al.*, 2002). It must be noted that at neutral pH no calcium burst is produced (Viladevall *et al.*, 2004), so calcineurin, which plays a major role in *PHO89* alkaline induction, won't be induced. Additionally, Snf1 activity is also pH-dependent, being 3 times more active at pH 8 that at pH 7 (Hong and Carlson, 2007). These additional pathways governing *PHO89* transcription would ensure its expression on strictly alkaline conditions, near the optimal pH of the transporter, and coinciding with a decrease in the transport rate of phosphate by Pho84.

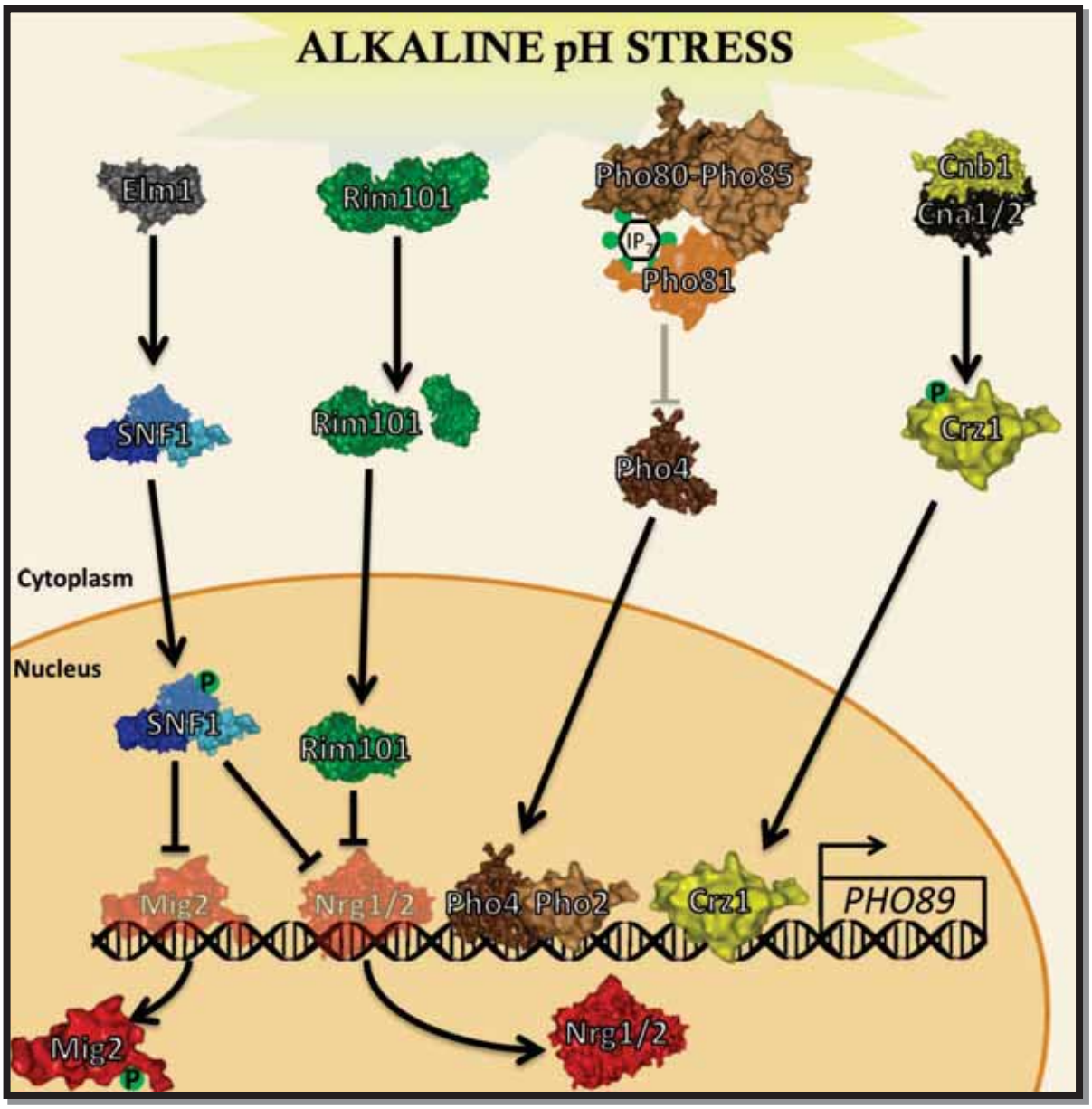

Figure 28. Schematic depiction of regulatory inputs acting on *PHO89* promoter upon high pH stress described in this work. Elements upstream of Rim101, Pho80-Pho85, or calcineurin are described in Introduction.

7. Coordinated expression of Pho89 and Ena1 allows functional coupling of both transporters

The previously documented resemblances between the Na^+-ATPase-encoding gene *ENA1* and *PHO89* induction under alkaline conditions (Serrano *et al.*, 2002) prompted us to look for DNA binding sites for transcription factors known to regulate *ENA1* (table 3). This led to the unravelling of the regulatory network described above, involving calcineurin, Rim101, Snf1, Mig2, and Nrg1/Nrg2, which is essentially identical to that reported several years ago for *ENA1* (Platara *et al.*, 2006). This high degree of similarity suggested the possibility of synchronous expression after alkaline pH stress and maybe some kind of functional relationship.

7.1. The *PHO89* promoter is not salt responsive

Exposure to high concentrations of salt triggers the activation of the calcineurin and Snf1 pathways (Hong and Carlson, 2007; Nakamura *et al.*, 1993). In addition, a severe salt stress induces *ENA1* through Sko1 inactivation and calcineurin activation (Márquez and Serrano, 1996; Mendoza *et al.*, 1994). Taking into account our previous observations that calcineurin and Snf1 induce *PHO89* expression during alkaline pH stress and the similarities between *PHO89* and *ENA1* regulation under this condition we wondered if the *PHO89* promoter was also responsive to salt stress. To verify this point, we performed a β-galactosidase activity assay to monitor *PHO89* promoter activity under salt stress conditions. We also employed the *ENA1* promoter (plasmid pKC201) and a synthetic tandem arrangement of CDREs (plasmid pAMS366) as positive controls.

As shown in figure 24, the wild type strain harboring the pPHO89-LacZ plasmid did not display any significant increase in β-galactosidase activity when exposed to mild or severe salt stress (0.4 M and 0.8 M NaCl, respectively), especially when compared to alkaline stress. In contrast, the *ENA1* promoter responded to both salt stresses, although not as strongly as to high pH conditions.

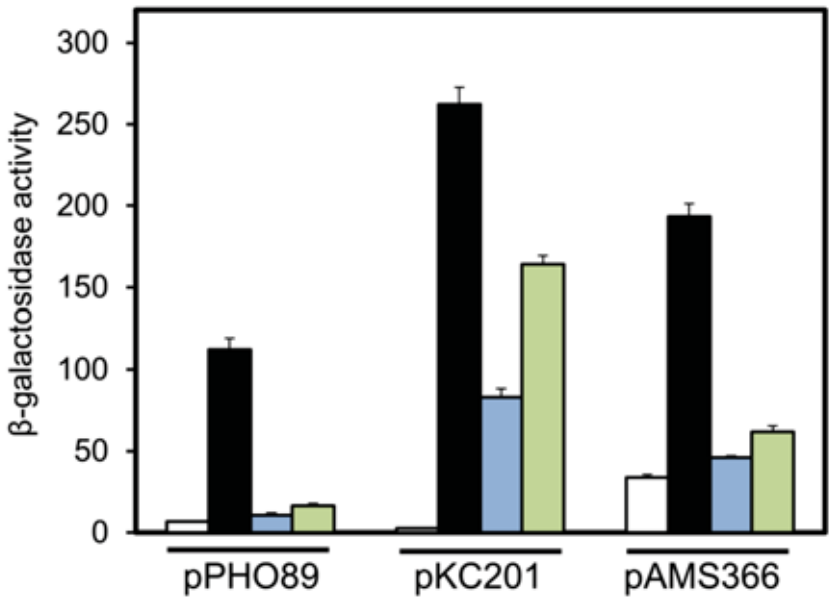

Figure 24. Wild type strain DBY746 was transformed with pPHO89-LacZ, pKC201 (carrying the entire *ENA1* promoter fused to *lacZ*) or pAMS366 (in which *lacZ* is driven by a 4x-CDRE tandem). Cells were subjected to high pH stress for 90 min (pH 8.0, black bars), 0.4 M NaCl for 60 min (blue bars) or 0.8 M NaCl for 90 min (green bars), prior determination of β-galactosidase activity. Data correspond to the mean ± SEM for 6 determinations.

The lack of *PHO89* induction under salt stress may be attributable to the few differences between its regulation and that of *ENA1*. For instance, it has never been described any direct involvement of the *PHO* pathway in the response to salt stress. This suggests that *PHO89* is not upregulated by Pho4 in this circumstance, thus lacking an important positive input. Additionally, even though exposure to high concentrations of salt activates Snf1, this kinase is not translocated into the nucleus, explaining why Mig1 does not become phosphorylated by salt stress (Hong and Carlson, 2007; McCartney and Schmidt, 2001). Since it is Mig2, and not Mig1, the factor repressing the *PHO89* promoter, we wanted to verify if this repressor was regulated by phosphorylation in cells exposed to salt stress. As shown in figure 25, a salt stress does not trigger the mobility shift characteristic of the phosphorylated forms of Mig2 observed after alkaline pH stress. Thus, this suggests that under conditions of stress by sodium cations *PHO89* is not upregulated by Snf1.

In summary, even though severe salt stress activates calcineurin (Denis and Cyert, 2002; Matsumoto *et al.*, 2002), either the potency of the signal is not sufficient to activate *PHO89* and/or the absence of additional regulatory inputs (e.g. activation of Pho4 and inhibition of Mig2) impedes its induction. Because, as far as we know, salt stress does not imply any impairment in phosphate acquisition, from a functional point of view the

expression of a high-affinity phosphate transporter does not seem necessary in these conditions. In fact, as Pho89 cotransports phosphate with sodium, its activity could be even detrimental in conditions of high extracellular sodium concentrations.

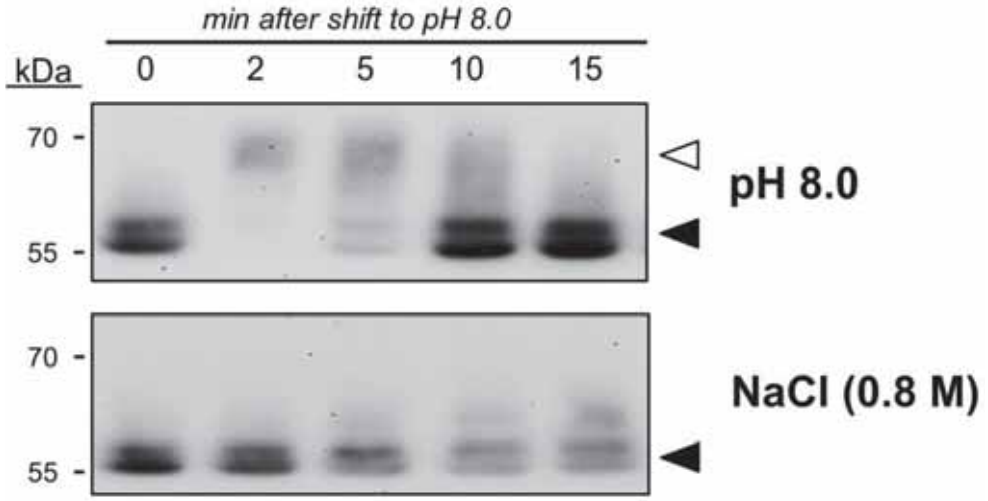

Figure 25. After exposing SP011 cells (*MIG2-3xHA*) to pH 8.0 or 0.8 M NaCl for the indicated times, extracts were prepared and subjected to 10% SDS-PAGE. Immunoblots were performed using anti-HA antibodies. The empty triangle denotes slow migrating (phosphorylated) species.

7.2. Pho89 and Ena1 show similar expression profiles under alkaline pH stress

In order to compare the kinetics of expression between *PHO89* and *ENA1* we performed a qRT-PCR to quantify *PHO89* mRNA, whereas for *ENA1* we used data from a Genomic Run-On (GRO) experiment performed in the same experimental conditions in collaboration with J. E. Perez-Ortín laboratory (Canadell *et al.*, 2015b). As shown in figure 26a, in both cases the mRNA levels increased rapidly after the induction, with a peak around 10-15 min, and then decreased nearly in parallel.

Next, we wanted to compare the kinetics of their protein accumulation. To that end, we introduced a C-terminally HA-tagged version of Ena1 to its own chromosomal locus and monitored its expression after alkaline stress. The immunoblot signals of this experiment and those of the previous experiments with the tagged version of Pho89 in YPD were integrated to calculate their relative protein levels (figure 26b). Similarly to what we observed for the mRNA levels, Pho89 and Ena1 display an early peak of accumulation (30-60 min). However, the subsequent decrease is more pronounced in Pho89, since 180 min after the alkaline

induction there is still a significant amount of Ena1 but Pho89 has returned already to its baseline level. These results indicate that both genes are coordinately expressed upon alkaline stress conditions and that their products appear in the cell at the same time.

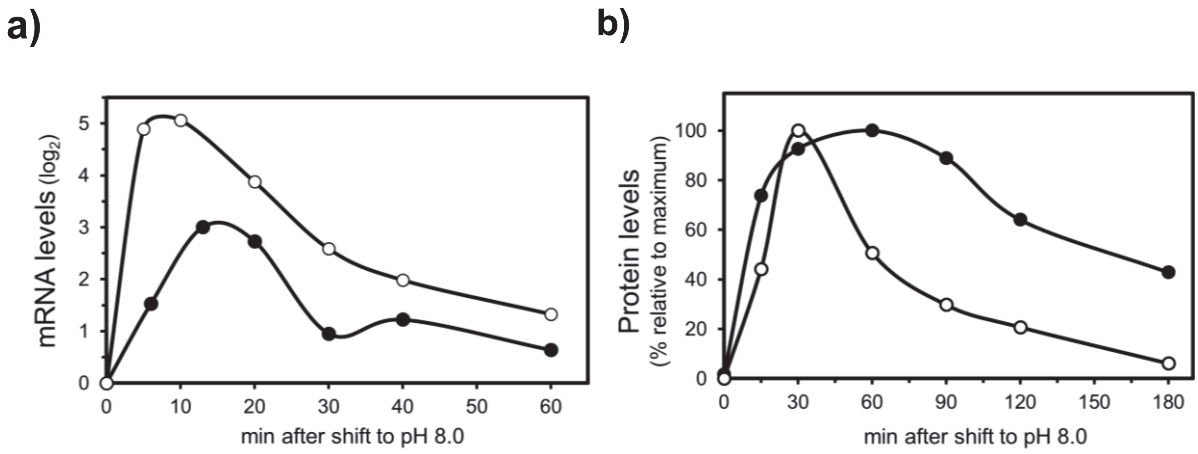

Figure 26. Time-course for *ENA1* (closed circles) and *PHO89* (open circles) mRNAs (a) or protein accumulation (b) after shifting cells from pH 5.5 to 8.0. Relative *PHO89* levels were calculated from a qRT-PCR performed following the same conditions as in figure 7a and *ENA1* levels are a representation of data published in Canadell *et al.*, 2015b. Relative Pho89 levels were calculated from 3 immunoblot experiments and Ena1 levels from a representative experiment using GelAnalyzer software after background subtraction.

7.3. The absence of Ena1 strongly impairs growth under alkaline conditions of a *pho84* mutant strain

Our previous observations that *PHO89* and *ENA1* share multiple regulatory pathways and that their coordinated expression occurs at high pH (when Pho89 is active), but not under salt stress conditions, prompted us to consider the existence of a functional link between both activities. To test this possibility, we decided to compare growth rates between mutant strains lacking Pho89, Pho84, Ena1, and their combinations at different pH and phosphate concentrations. In order to create an environment where Pho89 activity was indispensable, we were forced to include the *pho84* mutation, since this alternative high-affinity phosphate transporter was described to mediate phosphate transport, up to some extent, even at alkaline pH (Martinez and Persson, 1998; Zvyagilskaya *et al.*, 2008). Thus, due to the downregulation of the low-affinity phosphate transport system caused by the activation of the *PHO* pathway (see section 3.3 of Introduction), a *pho84* strain would rely only on Pho89 for phosphate import under alkaline or low phosphate conditions.

As shown in figure 27, the absence of Pho89 did not impair growth at low phosphate or mild alkaline pH conditions when compared to the wild type strain, probably due to Pho84 activity over this range of pH. In contrast, the *pho84* mutant strain displayed a severe growth deficiency in the presence of limiting amounts of phosphate at standard (acidic) pH, but its growth improved at higher pH. Since this strain only possesses Pho89 as a phosphate transporter in low phosphate or alkaline pH conditions, it is not surprising that it proliferates better at higher pH, corresponding with higher Pho89 expression and activity. As expected, further deletion of *PHO89* in this strain blocked proliferation on either phosphate starvation or alkaline pH conditions. Remarkably, elimination of the *ENA* cluster in a *pho84* background mimics the effect of mutating *PHO89*. Therefore, in a situation where Pho89 is the only phosphate transporter, abolishing the ability to extrude Na^+ cations blocks proliferation, thus phenocopying the situation of full deficiency of high-affinity phosphate transporters.

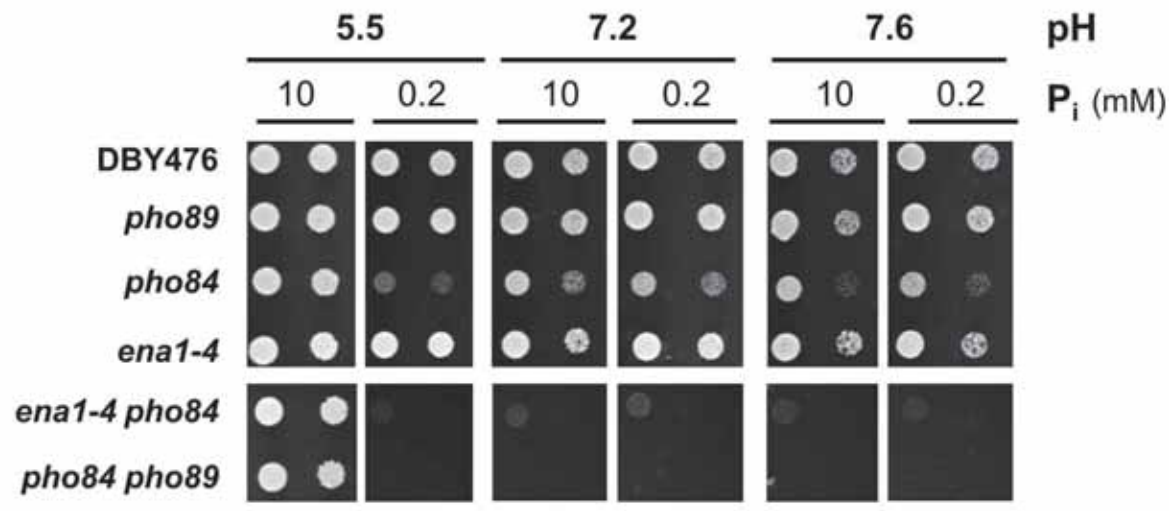

Figure 27. The indicated strains at OD_{660} of 0.05, plus a 10-fold dilution, were spotted on YNB-based medium (lacking phosphate and sodium) agar plates, supplemented with 5 mM NaCl and the indicated amounts of potassium phosphate, and adjusted to the different pHs. Growth was monitored after 3 days.

7.4. Unrestricted Pho89 activity could lead to toxic sodium accumulation

Shortly after the discovery of *PHO89*, Persson and coworkers postulated that the Na^+-ATPase activity of Ena1 could be providing the motive force for Na^+-P_i cotransport through Pho89 (Persson *et al.*, 1999). This would mean that, without this Na^+ extrusion activity, Pho89 would be unable to transport phosphate properly. However, our plates in figure 27 contained 5 mM Na^+, an external concentration described to drive a Pho89-mediated phosphate transport near to maximal (Martinez and Persson, 1998), thus making unlikely that external Na^+ could be the limiting factor for Pho89-mediated Pi uptake. Even so, we decided to test the effect of a lower external sodium concentration on the proliferation of the same mutant strains. To this end, we performed liquid growth assays at pH 7.2 in the presence of 5 mM and 0.2 mM NaCl with either 5 mM or 0.2 mM P_i, and calculated the 5/0.2 mM Na^+ growth ratio. In these conditions, the growth rates of the *ena1*, *pho84*, and *ena1 pho89* strains were not substantially different comparing 5 and 0.2mM external Na^+ at either high or low phosphate concentrations (figure 28). In contrast, the *ena1 pho84* strain grew much worse at 5 mM Na^+ than at 0.2 mM, regardless of phosphate availability. This result raised the

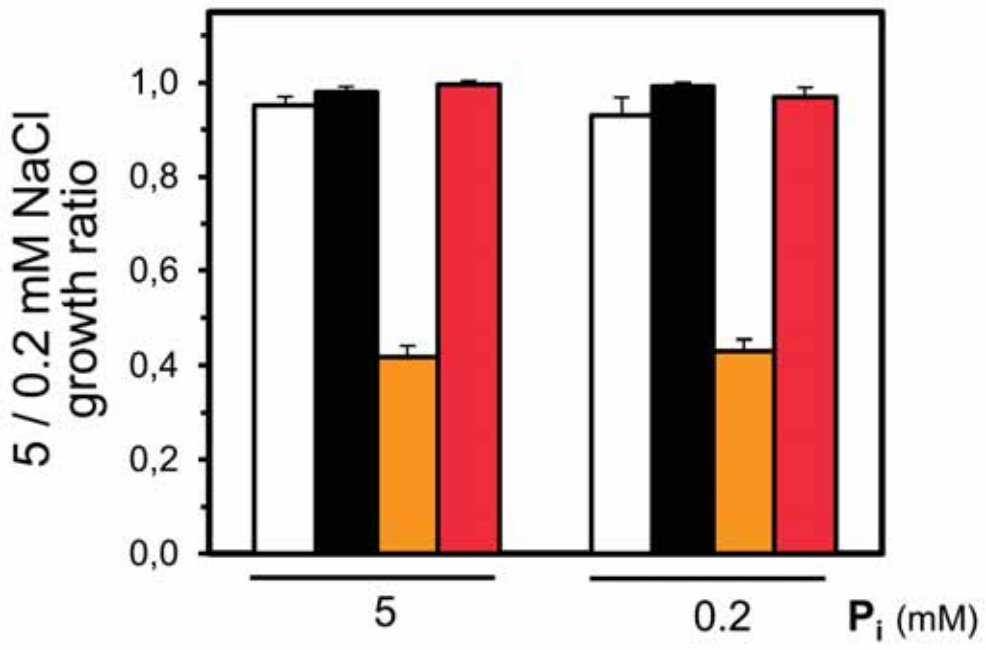

Figure 28. Strains ONA1 (*pho84,* white bars), RH16.6 (*ena1*, black bars), ASC17 (*ena1 pho84*, orange bars), and ASC30 (*ena1 pho89*, red bars) were grown in liquid media (adjusted to pH 7.2) containing the indicated concentrations of P_i and either 0.2 or 5 mM NaCl. The OD_{650} was determined after 47 h and the 5 mM / 0.2 mM NaCl absorbance ratio calculated. Data are mean ± SEM from 5 to 9 experiments.

possibility that the growth defect of the *ena1 pho84* strain at alkaline pH is not caused by the inability of Pho89 to uptake phosphate as a result of the absence of a Na^+ gradient across the plasma membrane, but instead because of an intracellular accumulation of toxic Na^+ cations accompanying the P_i influx, which cannot be eliminated without Ena1 activity. If this would be the case, the intracellular sodium concentration of the *ena1 pho84* strain should increase over time in parallel with the uptake of phosphate by Pho89. To test this possibility, we conducted intracellular Na^+ measurements in strains growing at pH 7.8 in a medium containing 0.2 mM P_i and 5 mM Na^+. Cultures were pre-incubated for 1 hour in the same medium at pH 5.5 in order to downregulate the low-affinity phosphate transport system, with the intention that when cells were shifted to alkaline conditions only the high-affinity transporters would mediate phosphate uptake.

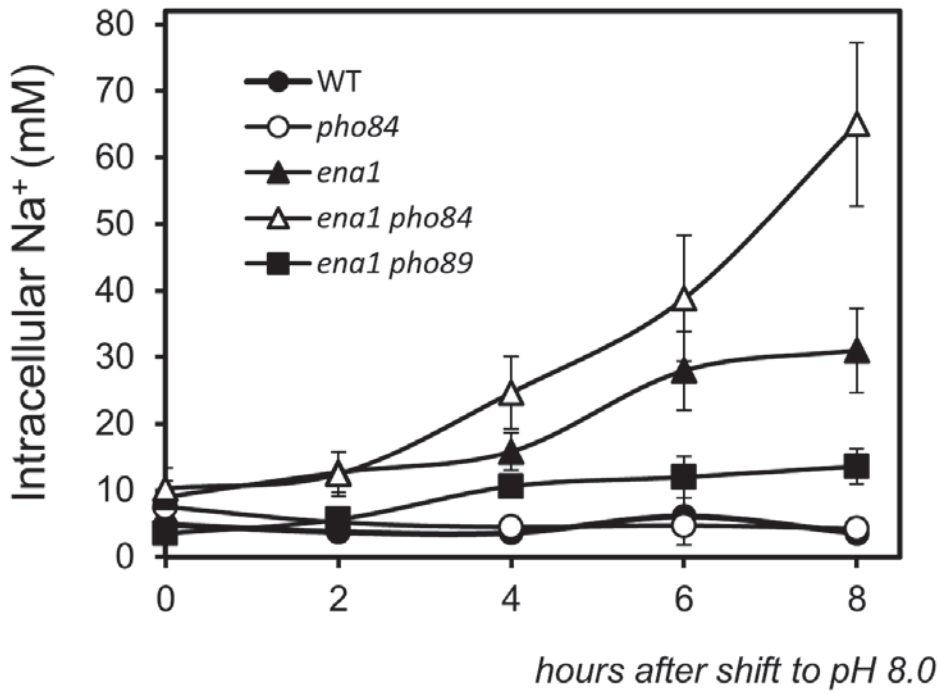

Figure 29. The indicated strains were shifted for 1 h to low P_i (0.2 mM) medium at pH 5.5 and then shifted to pH 7.8, always in the presence of 5 mM NaCl. Samples were taken at the indicated periods and processed for intracellular Na^+ content. Data are mean ± SEM from 4 to 8 independent experiments.

As shown in figure 29, all the strains tested exhibited an initial intracellular Na^+ concentration between 5 and 10 mM. The wild type and *pho84* strains (the ones possessing *ENA1*) did not accumulate any significant amount of sodium (< 5 mM) even 8 hours after the shift to alkaline conditions. Mutation of *ENA1* caused a moderate increase in sodium accumulation (31 mM) over time. Remarkably, the *ena1 pho84* mutant strain shows the

highest Na^+ accumulation (65 mM), twice as much as the *ena1* strain at 8 hours after the alkaline induction. On the other hand, the *ena1 pho89* strain accumulated much less intracellular sodium (14 mM), clearly below the levels measured for the *ena1* strain. The differences observed between the intracellular Na^+ accumulated by strains *ena1 pho84* and *ena1 pho89* point to the fact that Pho89 activity is indeed responsible for this accumulation. Interestingly, it was reported that a wild type strain continuously growing in the presence of 500 mM NaCl accumulated as much as 90 mM of Na^+ (Ros *et al.*, 1998). An external concentration of 500 mM Na^+ (a 100-fold higher than the one used in our experiment) already impairs growth of a wild type strain; thus, it is conceivable that the sensitive phenotype displayed by the *ena1 pho84* strain is caused by the continued accumulation of this toxic cation.

In summary, these results indicate that, in a situation where phosphate uptake is dependent exclusively on Pho89 (i.e. the *pho84* strain under alkaline conditions), the absence of the Na^+-ATPase Ena1 provokes a severe growth defect that correlates with the accumulation of high concentrations of intracellular Na^+. This accumulation might entail severe effects on cell physiology, not only because sodium is a recognized toxic cation, but also because an increase in its intracellular levels could reverse the external/internal cation gradient necessary for phosphate cotransport via Pho89, thus blocking further phosphate uptake. Therefore, we postulate that the sodium efflux activity of Ena1 may serve as a detoxifying mechanism to counteract the sodium uptake through Pho89, which may otherwise become detrimental. This would provide a physiological meaning for the parallelisms observed between the mechanisms regulating both Ena1 and Pho89 induction under high pH stress, ensuring a coordinated expression to allow the functional coupling of both transporters.

The Ena1-Pho89 functional coupling described in this work might not be restricted to *S. cerevisiae*. *ENA* genes have been found on 56 of the 65 sequenced genomes of fungi, as well as in several protozoa and bryophytes (Rodríguez-Navarro and Benito, 2010). Studies performed in several of these species, such as *C. albicans*, *N. crassa* or *A. nidulans*, revealed that their ENA ATPases were weakly expressed in normal growth conditions but upregulated by salt and/or alkaline stresses (Benito *et al.*, 2000; Bensen *et al.*, 2004; Spielvogel *et al.*, 2008). In fact, it is known that *CaENA2* and *CaENA21* are regulated by *Ca*Rim101 and

*Ca*Crz1/*Ca*Crz2 upon alkaline stress (Kullas *et al.*, 2007). Additionally, deletion of the *ENA* gen in *Ustilago maydis* or *Cryptococcus neoformans* confers an alkali-sensitive phenotype (Benito *et al.*, 2009; Idnurm *et al.*, 2009). On the other hand, Pho89-like transporters (i.e. type-III Na^+-P_i cotransporters) are widely spread among eukaryotes (Werner and Kinne, 2001). Most studies regarding Pho89 homologues have focused on phosphate and sodium homeostasis, so there is not much information about the role of this transporter in other situations. However, a relationship of Pho89 with the response to alkaline stress has been described in *Candida albicans*. Remarkably, *CaPHO89* is strongly induced in alkaline conditions and part of this induction is dependent on *Ca*Rim101 (Bensen *et al.*, 2004). While this work was in progress, further studies in *C. albicans* revealed that *CaPHO89* alkaline upregulation was also dependent on calcineurin-*Ca*Crz1, activated by a rapid burst of cytosolic calcium from the external medium (Wang *et al.*, 2011). It is worth noting that both *Ca*Rim101 and calcineurin are involved in the transcriptional response required for *C. albicans* virulence in alkaline niches (Bader *et al.*, 2006; Ramon *et al.*, 1999). Taking all this into account, it is not farfetched to postulate that the functional and regulatory coupling between Ena1 and Pho89 reported in this work may not be exclusive of *S. cerevisiae*.

7.5. Gene coregulation analysis as a tool to study protein function

The response to alkaline stress encompasses the activation of a multitude of different pathways, in some cases apparently unrelated (see section 2 of Introduction). These signaling pathways lead to remodeling of gene expression in order to cope with high pH stress; therefore, it is not surprising that the transcription of a given gene may be regulated by more than one pathway. In this work we have observed that the complex regulation of Pho89 under alkaline conditions is nearly identical to that previously described for Ena1 (Platara *et al.*, 2006), and that this synchronic expression could allow their functional coupling. Therefore, it is possible that the analysis of coregulation on a transcriptomic level could, in some cases, unravel other functional pairs.

In a recent work carried out in our laboratory we examined different sets of transcriptomic data obtained from comparisons between the wild type strain and *msn2 msn4*,

snf1, *cnb1/crz1*, *rim101,* or *slt2* (Serra-Cardona *et al.* 2015). Up to 349 genes were upregulated by at least one of the mentioned pathways, being Msn2/Msn4 the transcription factor with a wider impact, followed by Snf1. Around 17% of these genes are controlled by more than one factor, and 7 genes related to carbohydrate metabolism are coregulated by Snf1, Msn2/Msn4, and calcineurin. In contrast, the subsets of genes under the influence of Rim101 or Slt2 are less interconnected with other pathways (Serra-Cardona *et al.* 2015). Further study of these networks and the subset of genes they regulate could reveal new roles for the proteins expressed after high pH conditions and give insight into the mechanisms by which the budding yeast copes with alkaline stress.

CONCLUSIONS

1) Pho89 is rapidly but transiently induced by alkaline conditions, with a maximum accumulation at 30 minutes after the stress. This response is driven by a complex regulatory network comprised of Pho4, calcineurin, Rim101, and Snf1.

2) Pho89 expression under conditions of phosphate scarcity is weaker and occurs later when compared to high pH stress. On the other hand, the expression profile of Pho84 is nearly identical between both situations and solely dependent on Pho4.

3) The calcineurin pathway strongly upregulates *PHO89* after alkaline stress through the Crz1 transcription factor. This effect occurs mainly at the CDRE2, at positions -273 to -267, and Crz1 is bound to this regulatory element shortly and transiently (2-10 min) after the stress.

4) The absence of Rim101 causes a downregulation of Pho89 alkaline induction especially evident at a long-term. Although both Nrg1 and Nrg2 exert some degree of repression on *PHO89*, only Nrg1 seems to be regulated in a Rim101-dependent manner.

5) The Snf1 pathway mediates a positive effect on *PHO89* expression under alkaline conditions via relieving the repression by Mig2. In contrast, Mig1 plays a secondary role in the repression of this gene.

6) The Snf1 kinase is quickly but briefly phosphorylated after high pH stress. Mig1 and Mig2 also present a phosphorylated state for a short time in these same conditions, and this process is dependent on Snf1. The phosphorylated forms of these repressors are coincident with their cytosolic localization after high pH stress.

7) The *reg1* mutation results in the phosphorylation of Snf1 even at acidic pH and in constitutive nuclear exclusion of Mig2. This same strain exhibits increased levels of *PHO89* under alkaline conditions, particularly at a long-term.

8) When compared to the wild type, the *reg1* strain displays a drastic upregulation of Pho89 long after the alkaline stress, probably caused by the constitutive inhibition of the Mig repressors. We also speculate that Snf1 and Reg1 might be controlling Pho89 stability through the regulation of arrestins, as described for other plasma membrane transporters.

9) The regulatory network responsible for the alkaline induction of Pho89 reported in this work is nearly identical to the one described for Ena1. These similarities lead to a synchronous expression of both proteins after high pH stress.

10) In a situation where phosphate uptake depends exclusively on Pho89 (i.e. a *pho84* strain under alkaline conditions) the absence of *ENA1* strongly impairs growth. In addition, this same strain shows a marked sensitive phenotype to low amounts of external Na^+.

11) When growing under low phosphate and alkaline conditions, *ena1-4 pho84* cells rapidly accumulate high amounts of intracellular sodium as a result of Pho89 activity. We postulate that in this situation the coregulation of Pho89 and Ena1 allows their functional coupling: the Na^+ extrusion capacity of Ena1 could serve as a detoxifying mechanism to allow persistent Pho89-mediated Na^+/P_i cotransport.

REFERENCES

Adams, A., Gottschling, D.E., Kaiser, C.A., and Stearns, T. (1997). Methods in Yeast Genetics (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, NY).

Alepuz, P.M., Cunningham, K.W., and Estruch, F. (1997). Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated ENA1 gene. Mol. Microbiol. *26*, 91–98.

Alvaro, C.G., O'Donnell, A.F., Prosser, D.C., Augustine, A.A., Goldman, A., Brodsky, J.L., Cyert, M.S., Wendland, B., and Thorner, J. (2014). Specific α-arrestins negatively regulate Saccharomyces cerevisiae pheromone response by down-modulating the G-protein-coupled receptor Ste2. Mol. Cell. Biol. *34*, 2660–2681.

Arima, K., Oshima, T., Kubota, I., Nakamura, N., Mizunaga, T., and Toh-e, A. (1983). The nucleotide sequence of the yeast PHO5 gene: a putative precursor of repressible acid phosphatase contains a signal peptide 1 488. Nucleic Acids Res. *11*, 1657–1672.

Arino, J. (2010). Integrative responses to high pH stress in S. cerevisiae. OMICS *14*, 517–523.

Ariño, J., Ramos, J., and Sychrová, H. (2010). Alkali metal cation transport and homeostasis in yeasts. Microbiol. Mol. Biol. Rev. *74*, 95–120.

Auesukaree, C., Homma, T., Kaneko, Y., and Harashima, S. (2003). Transcriptional regulation of phosphate-responsive genes in low-affinity phosphate-transporter-defective mutants in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. *306*, 843–850.

Bader, T., Schröppel, K., Bentink, S., Agabian, N., Köhler, G., and Morschhäuser, J. (2006). Role of calcineurin in stress resistance, morphogenesis, and virulence of a Candida albicans wild-type strain. Infect. Immun. *74*, 4366–4369.

Barbaric, S., Münsterkötter, M., Goding, C., and Hörz, W. (1998). Cooperative Pho2-Pho4 interactions at the PHO5 promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol. Cell. Biol. *18*, 2629–2639.

Barreto, L., Canadell, D., Valverde-Saubí, D., Casamayor, A., and Ariño, J. (2012). The short-term response of yeast to potassium starvation. Environ. Microbiol. *14*, 3026–3042.

Barwell, K.J., Boysen, J.H., Xu, W., and Mitchell, A.P. (2005). Relationship of DFG16 to the Rim101p pH response pathway in Saccharomyces cerevisiae and Candida albicans. Eukaryot. Cell *4*, 890–899.

Batiza, A.F., Schulz, T., and Masson, P.H. (1996). Yeast respond to hypotonic shock with a calcium pulse. J. Biol. Chem. *271*, 23357–23362.

Becuwe, M., Vieira, N., Lara, D., Gomes-Rezende, J., Soares-Cunha, C., Casal, M., Haguenauer-Tsapis, R., Vincent, O., Paiva, S., and Léon, S. (2012). A molecular switch on an arrestin-like protein relays glucose signaling to transporter endocytosis. J. Cell Biol. *196*, 247–259.

Benito, B., Garciadeblás, B., and Rodríguez-Navarro, A. (2000). Molecular cloning of the calcium and sodium ATPases in Neurospora crassa. Mol. Microbiol. *35*, 1079–1088.

Benito, B., Garciadeblás, B., Pérez-Martín, J., and Rodríguez-Navarro, A. (2009). Growth at high pH and sodium and potassium tolerance in media above the cytoplasmic pH depend on ENA ATPases in Ustilago maydis. Eukaryot. Cell *8*, 821–829.

Bensen, E.S., Martin, S.J., Li, M., Berman, J., and Davis, D.A. (2004). Transcriptional profiling in Candida albicans reveals new adaptive responses to extracellular pH and functions for Rim101p. Mol. Microbiol. *54*, 1335–1351.

Berkey, C.D., Vyas, V.K., and Carlson, M. (2004). Nrg1 and nrg2 transcriptional repressors are differently regulated in response to carbon source. Eukaryot. Cell *3*, 311–317.

Beullens, M., Mbonyi, K., Geerts, L., Gladines, D., Detremerie, K., Jans, A.W., and Thevelein, J.M. (1988). Studies on the mechanism of the glucose-induced cAMP signal in glycolysis and glucose repression mutants of the yeast Saccharomyces cerevisiae. Eur. J. Biochem. *172*, 227–231.

Blaiseau, P.L., Lesuisse, E., and Camadro, J.M. (2001). Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. J. Biol. Chem. *276*, 34221–34226.

Boer, V.M., de Winde, J.H., Pronk, J.T., and Piper, M.D.W. (2003). The genome-wide transcriptional responses of Saccharomyces cerevisiae grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J. Biol. Chem. *278*, 3265–3274.

Bojunga, N., and Entian, K.D. (1999). Cat8p, the activator of gluconeogenic genes in Saccharomyces cerevisiae, regulates carbon source-dependent expression of NADP-dependent cytosolic isocitrate dehydrogenase (Idp2p) and lactate permease (Jen1p). Mol. Gen. Genet. *262*, 869–875.

Bonilla, M., and Cunningham, K.W. (2003). Mitogen-activated protein kinase stimulation of Ca(2+) signaling is required for survival of endoplasmic reticulum stress in yeast. Mol.Biol.Cell *14*, 4296–4305.

Bonilla, M., Nastase, K.K., and Cunningham, K.W. (2002). Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J. *21*, 2343–2353.

Bostian, K.A., Lemire, J.M., Cannon, L.E., and Halvorson, H.O. (1980). In vitro synthesis of repressible yeast acid phosphatase: identification of multiple mRNAs and products 2 489 490. Proc.Natl.Acad.Sci.U.S A *77*, 4504–4508.

Boustany, L.M., and Cyert, M.S. (2002). Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. Genes Dev. *16*, 608–619.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115–132.

Brazas, R.M., and Stillman, D.J. (1993). The Swi5 zinc-finger and Grf10 homeodomain proteins bind DNA cooperatively at the yeast HO promoter. Proc. Natl. Acad. Sci. U. S. A. *90*, 11237–11241.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. Science *259*, 1760–1763.

Bun-Ya, M., Nishimura, M., Harashima, S., and Oshima, Y. (1991). The PHO84 gene of Saccharomyces cerevisiae encodes an inorganic phosphate transporter. Mol. Cell. Biol. *11*, 3229–3238.

Bun-ya, M., Shikata, K., Nakade, S., Yompakdee, C., Harashima, S., and Oshima, Y. (1996). Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. Curr. Genet. *29*, 344–351.

Caddick, M.X., Brownlee, A.G., and Arst, H.N. (1986). Regulation of gene expression by pH of the growth medium in Aspergillus nidulans. Mol. Gen. Genet. *203*, 346–353.

Calcagno-Pizarelli, A.M., Negrete-Urtasun, S., Denison, S.H., Rudnicka, J.D., Bussink, H.-J., Munera-Huertas, T., Stanton, L., Hervas-Aguilar, A., Espeso, E.A., Tilburn, J., et al. (2007). Establishment of the Ambient pH Signaling Complex in Aspergillus nidulans: PalI Assists Plasma Membrane Localization of PalH. Eukaryot. Cell *6*, 2365–2375.

Camblong, J., Iglesias, N., Fickentscher, C., Dieppois, G., and Stutz, F. (2007). Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in S. cerevisiae. Cell *131*, 706–717.

Camblong, J., Beyrouthy, N., Guffanti, E., Schlaepfer, G., Steinmetz, L.M., and Stutz, F. (2009). Trans-acting antisense RNAs mediate transcriptional gene cosuppression in S. cerevisiae. Genes Dev. *23*, 1534–1545.

Cameroni, E., Hulo, N., Roosen, J., Winderickx, J., and De Virgilio, C. (2004). The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms. Cell Cycle *3*, 462–468.

Canadell, D., González, A., Casado, C., and Ariño, J. (2015a). Functional interactions between potassium and phosphate homeostasis in Saccharomyces cerevisiae. Mol. Microbiol. *95*, 555–572.

Canadell, D., García-Martínez, J., Alepuz, P., Pérez-Ortín, J.E., and Ariño, J. (2015b). Impact of high pH stress on yeast gene expression: A comprehensive analysis of mRNA turnover during stress responses. Biochim. Biophys. Acta *1849*, 653–664.

Carlson, M., and Botstein, D. (1982). Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase. Cell *28*, 145–154.

Carlson, M., Osmond, B.C., and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. Genetics *98*, 25–40.

Casado, C., Yenush, L., Melero, C., Ruiz Mdel, C., Serrano, R., Perez-Valle, J., Arino, J., and Ramos, J. (2010). Regulation of Trk-dependent potassium transport by the calcineurin pathway involves the Hal5 kinase. FEBS Lett. *584*, 2415–2420.

Casado, C., González, A., Platara, M., Ruiz, A., and Ariño, J. (2011). The role of the protein kinase A pathway in the response to alkaline pH stress in yeast. Biochem. J. *438*, 523–533.

Casamayor, A., Serrano, R., Platara, M., Casado, C., Ruiz, A., and Ariño, J. (2012). The role of the Snf1 kinase in the adaptive response of Saccharomyces cerevisiae to alkaline pH stress. Biochem. J. *444*, 39–49.

Castrejon, F., Gomez, A., Sanz, M., Duran, A., and Roncero, C. (2006). The RIM101 pathway contributes to yeast cell wall assembly and its function becomes essential in the absence of mitogen-activated protein kinase Slt2p. Eukaryot.Cell *5*, 507–517.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S., and Young, R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. Mol.Biol.Cell *12*, 323–337.

Celenza, J.L., and Carlson, M. (1986). A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science *233*, 1175–1180.

Celenza, J.L., and Carlson, M. (1989). Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol. Cell. Biol. *9*, 5034–5044.

Chen, O.S., Crisp, R.J., Valachovic, M., Bard, M., Winge, D.R., and Kaplan, J. (2004). Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis. J. Biol. Chem. *279*, 29513–29518.

Cockburn, M., Earnshaw, P., and Eddy, A.A. (1975). The stoicheiometry of the absorption of protons with phosphate and L-glutamate by yeasts of the genus Saccharomyces. Biochem. J. *146*, 705–712.

Cullen, P.J., and Sprague, G.F. (2000). Glucose depletion causes haploid invasive growth in yeast. Proc. Natl. Acad. Sci. U. S. A. *97*, 13619–13624.

Cunningham, K.W., and Fink, G.R. (1994). Calcineurin-dependent growth control in Saccharomyces cerevisiae mutants lacking PMC1, a homolog of plasma membrane Ca2+ ATPases. J.Cell Biol. *124*, 351–363.

Cunningham, K.W., and Fink, G.R. (1996). Calcineurin inhibits VCX1-dependent H+/Ca2+ exchange and induces Ca2+ ATPases in Saccharomyces cerevisiae. Mol. Cell. Biol. *16*, 2226–2237.

Cyert, M.S. (2001). Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. Annu.Rev.Genet. *35*, 647–672.

Cyert, M.S., and Thorner, J. (1992). Regulatory subunit (CNB1 gene product) of yeast Ca2+/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. *12*, 3460–3469.

Cyert, M.S., Kunisawa, R., Kaim, D., and Thorner, J. (1991). Yeast has homologs (CNA1 and CNA2 gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc. Natl. Acad. Sci. U. S. A. *88*, 7376–7380.

Daignan-Fornier, B., and Fink, G.R. (1992). Coregulation of purine and histidine biosynthesis by the transcriptional activators BAS1 and BAS2. Proc. Natl. Acad. Sci. U. S. A. *89*, 6746–6750.

Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R.D. (1994). Molecular characterization of a copper transport protein in S. cerevisiae: an unexpected role for copper in iron transport. Cell *76*, 393–402.

Davis, D. (2003). Adaptation to environmental pH in Candida albicans and its relation to pathogenesis. Curr. Genet. *44*, 1–7.

Davis, D., Wilson, R.B., and Mitchell, A.P. (2000). RIM101-dependent and-independent pathways govern pH responses in Candida albicans. Mol. Cell. Biol. *20*, 971–978.

Denis, V., and Cyert, M.S. (2002). Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. J.Cell Biol. *156*, 29–34.

Denison, S.H., Negrete-Urtasun, S., Mingot, J.M., Tilburn, J., Mayer, W.A., Goel, A., Espeso, E.A., Peñalva, M.A., and Arst, H.N. (1998). Putative membrane components of signal transduction pathways for ambient pH regulation in Aspergillus and meiosis in saccharomyces are homologous. Mol. Microbiol. *30*, 259–264.

DeVit, M.J., and Johnston, M. (1999). The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae. Curr. Biol. *9*, 1231–1241.

DeVit, M.J., Waddle, J.A., and Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. Mol. Biol. Cell *8*, 1603–1618.

Díez, E., Alvaro, J., Espeso, E.A., Rainbow, L., Suárez, T., Tilburn, J., Arst, H.N., and Peñalva, M.A. (2002). Activation of the Aspergillus PacC zinc finger transcription factor requires two proteolytic steps. EMBO J. *21*, 1350–1359.

Donella-Deana, A., Ostojić, S., Pinna, L.A., and Barbarić, S. (1993). Specific dephosphorylation of phosphopeptides by the yeast alkaline phosphatase encoded by PHO8 gene. Biochim. Biophys. Acta *1177*, 221–228.

Drgonová, J., Drgon, T., Tanaka, K., Kollár, R., Chen, G.C., Ford, R.A., Chan, C.S., Takai, Y., and Cabib, E. (1996). Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science *272*, 277–279.

Dubacq, C., Chevalier, A., and Mann, C. (2004). The protein kinase Snf1 is required for tolerance to the ribonucleotide reductase inhibitor hydroxyurea. Mol. Cell. Biol. *24*, 2560–2572.

Dunn, T., Gable, K., and Beeler, T. (1994). Regulation of cellular Ca2+ by yeast vacuoles. J. Biol. Chem. *269*, 7273–7278.

Dupres, V., Alsteens, D., Wilk, S., Hansen, B., Heinisch, J.J., and Dufrêne, Y.F. (2009). The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. Nat. Chem. Biol. *5*, 857–862.

Errede, B., Cade, R.M., Yashar, B.M., Kamada, Y., Levin, D.E., Irie, K., and Matsumoto, K. (1995). Dynamics and organization of MAP kinase signal pathways. Mol. Reprod. Dev. *42*, 477–485.

Espeso, E.A., and Arst, H.N. (2000). On the mechanism by which alkaline pH prevents expression of an acid-expressed gene. Mol. Cell. Biol. *20*, 3355–3363.

Espeso, E.A., and Peñalva, M.A. (1996). Three binding sites for the Aspergillus nidulans PacC zinc-finger transcription factor are necessary and sufficient for regulation by ambient pH of the isopenicillin N synthase gene promoter. J. Biol. Chem. *271*, 28825–28830.

Estruch, F., and Carlson, M. (1993). Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of Saccharomyces cerevisiae. Mol. Cell. Biol. *13*, 3872–3881.

Farcasanu, I.C., Hirata, D., Tsuchiya, E., Nishiyama, F., and Miyakawa, T. (1995). Protein phosphatase 2B of Saccharomyces cerevisiae is required for tolerance to manganese, in blocking the entry of ions into the cells 1. Eur. J. Biochem. *232*, 712–717.

Fernández-Cid, A., Riera, A., Herrero, P., and Moreno, F. (2012). Glucose levels regulate the nucleo-mitochondrial distribution of Mig2. Mitochondrion *12*, 370–380.

Fisher, F., Jayaraman, P.S., and Goding, C.R. (1991). C-myc and the yeast transcription factor PHO4 share a common CACGTG-binding motif. Oncogene *6*, 1099–1104.

Futai, E., Maeda, T., Sorimachi, H., Kitamoto, K., Ishiura, S., and Suzuki, K. (1999). The protease activity of a calpain-like cysteine protease in Saccharomyces cerevisiae is required for alkaline adaptation and sporulation. Mol. Gen. Genet. *260*, 559–568.

Gaber, R.F., Styles, C.A., and Fink, G.R. (1988). TRK1 encodes a plasma membrane protein required for high-affinity potassium transport in Saccharomyces cerevisiae. Mol. Cell. Biol. *8*, 2848–2859.

Galindo, A., Hervás-Aguilar, A., Rodríguez-Galán, O., Vincent, O., Arst, H.N., Tilburn, J., and Peñalva, M.A. (2007). PalC, one of two Bro1 domain proteins in the fungal pH signalling pathway, localizes to cortical structures and binds Vps32. Traffic *8*, 1346–1364.

Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Pena, J.M., Francois, J., Nombela, C., and Arroyo, J. (2004). The global transcriptional response to transient cell wall damage in Saccharomyces cerevisiae and its regulation by the cell integrity signaling pathway. J. Biol. Chem. *279*, 15183–15195.

Garciadeblas, B., Rubio, F., Quintero, F.J., Banuelos, M.A., Haro, R., and Rodriguez-Navarro, A. (1993). Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. Mol.Gen.Genet. *236*, 363–368.

Garrett, S., Menold, M.M., and Broach, J.R. (1991). The Saccharomyces cerevisiae YAK1 gene encodes a protein kinase that is induced by arrest early in the cell cycle. Mol. Cell. Biol. *11*, 4045–4052.

Gauthier, S., Coulpier, F., Jourdren, L., Merle, M., Beck, S., Konrad, M., Daignan-Fornier, B., and Pinson, B. (2008). Co-regulation of yeast purine and phosphate pathways in response to adenylic nucleotide variations. Mol. Microbiol. *68*, 1583–1594.

Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature *425*, 737–741.

Ghillebert, R., Swinnen, E., De Snijder, P., Smets, B., and Winderickx, J. (2011). Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in Saccharomyces cerevisiae. Biochem. J. *434*, 243–251.

Giots, F., Donaton, M.C. V, and Thevelein, J.M. (2003). Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol. Microbiol. *47*, 1163–1181.

Goldman, A., Roy, J., Bodenmiller, B., Wanka, S., Landry, C.R., Aebersold, R., and Cyert, M.S. (2014). The calcineurin signaling network evolves via conserved kinase-phosphatase modules that transcend substrate identity. Mol. Cell *55*, 422–435.

Gomez-Raja, J., and Davis, D.A. (2012). The β-arrestin-like protein Rim8 is hyperphosphorylated and complexes with Rim21 and Rim101 to promote adaptation to neutral-alkaline pH. Eukaryot. Cell *11*, 683–693.

González, A., Casado, C., Ariño, J., and Casamayor, A. (2013). Ptc6 Is Required for Proper Rapamycin-Induced Down-Regulation of the Genes Coding for Ribosomal and rRNA Processing Proteins in S. cerevisiae. PLoS One *8*, e64470.

Goodman, J., and Rothstein, A. (1957). The active transport of phosphate into the yeast cell. J. Gen. Physiol. *40*, 915–923.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schüller, C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. *12*, 586–597.

Guo, W., Tamanoi, F., and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. Nat. Cell Biol. *3*, 353–360.

Hardie, D.G. (2011). AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev. *25*, 1895–1908.

Hardy, T.A., Huang, D., and Roach, P.J. (1994). Interactions between cAMP-dependent and SNF1 protein kinases in the control of glycogen accumulation in Saccharomyces cerevisiae. J. Biol. Chem. *269*, 27907–27913.

Haro, R., Garciadeblas, B., and Rodriguez-Navarro, A. (1991). A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. *291*, 189–191.

Haurie, V., Perrot, M., Mini, T., Jeno, P., Sagliocco, F., and Boucherie, H. (2000). The Transcriptional Activator Cat8p Provides a Major Contribution to the Reprogramming of Carbon Metabolism during the Diauxic Shift in Saccharomyces cerevisiae. J. Biol. Chem. *276*, 76–85.

Heath, V.L., Shaw, S.L., Roy, S., and Cyert, M.S. (2004). Hph1p and Hph2p, novel components of calcineurin-mediated stress responses in Saccharomyces cerevisiae. Eukaryot.Cell *3*, 695–704.

Hedbacker, and Carlson, M. (2006). Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. Eukaryot. Cell *5*, 1950–1956.

Hedbacker, K., Hong, S.-P., and Carlson, M. (2004a). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. Mol. Cell. Biol. *24*, 8255–8263.

Hedbacker, K., Townley, R., and Carlson, M. (2004b). Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. Mol. Cell. Biol. *24*, 1836–1843.

Hedges, D., Proft, M., and Entian, K.D. (1995). CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. *15*, 1915–1922.

Herrador, A., Herranz, S., Lara, D., and Vincent, O. (2010). Recruitment of the ESCRT machinery to a putative seven-transmembrane-domain receptor is mediated by an arrestin-related protein. Mol. Cell. Biol. *30*, 897–907.

Hervás-Aguilar, A., Galindo, A., and Peñalva, M.A. (2010). Receptor-independent Ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF. J. Biol. Chem. *285*, 18095–18102.

Hilioti, Z., Gallagher, D.A., Low-Nam, S.T., Ramaswamy, P., Gajer, P., Kingsbury, T.J., Birchwood, C.J., Levchenko, A., and Cunningham, K.W. (2004). GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. Genes Dev. *18*, 35–47.

Hirst, K., Fisher, F., McAndrew, P.C., and Goding, C.R. (1994). The transcription factor, the Cdk, its cyclin and their regulator: directing the transcriptional response to a nutritional signal. EMBO J. *13*, 5410–5420.

Hong, S.-P., and Carlson, M. (2007). Regulation of snf1 protein kinase in response to environmental stress. J. Biol. Chem. *282*, 16838–16845.

Hong, S.-P., Leiper, F.C., Woods, A., Carling, D., and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc. Natl. Acad. Sci. U. S. A. *100*, 8839–8843.

Hong, S.-P., Momcilovic, M., and Carlson, M. (2005). Function of mammalian LKB1 and Ca2+/calmodulin-dependent protein kinase kinase alpha as Snf1-activating kinases in yeast. J. Biol. Chem. *280*, 21804–21809.

Honigberg, S.M., and Lee, R.H. (1998). Snf1 kinase connects nutritional pathways controlling meiosis in Saccharomyces cerevisiae. Mol. Cell. Biol. *18*, 4548–4555.

Horak, J., and Wolf, D.H. (2001). Glucose-induced monoubiquitination of the Saccharomyces cerevisiae galactose transporter is sufficient to signal its internalization. J. Bacteriol. *183*, 3083–3088.

Hothorn, M., Neumann, H., Lenherr, E.D., Wehner, M., Rybin, V., Hassa, P.O., Uttenweiler, A., Reinhardt, M., Schmidt, A., Seiler, J., et al. (2009). Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science *324*, 513–516.

Huang, S., and O'Shea, E.K. (2005). A systematic high-throughput screen of a yeast deletion collection for mutants defective in PHO5 regulation. Genetics *169*, 1859–1871.

Huang, D., Farkas, I., and Roach, P.J. (1996). Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in Saccharomyces cerevisiae. Mol. Cell. Biol. *16*, 4357–4365.

Huang, D., Friesen, H., and Andrews, B. (2007). Pho85, a multifunctional cyclin-dependent protein kinase in budding yeast. Mol. Microbiol. *66*, 303–314.

Hürlimann, H.C., Stadler-Waibel, M., Werner, T.P., and Freimoser, F.M. (2007). Pho91 Is a vacuolar phosphate transporter that regulates phosphate and polyphosphate metabolism in Saccharomyces cerevisiae. Mol. Biol. Cell *18*, 4438–4445.

Huxley, C., Green, E.D., and Dunham, I. (1990). Rapid assessment of S. cerevisiae mating type by PCR. Trends Genet. *6*, 236.

Idnurm, A., Walton, F.J., Floyd, A., Reedy, J.L., and Heitman, J. (2009). Identification of ENA1 as a virulence gene of the human pathogenic fungus Cryptococcus neoformans through signature-tagged insertional mutagenesis. Eukaryot. Cell *8*, 315–326.

Igual, J.C., Johnson, A.L., and Johnston, L.H. (1996). Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. EMBO J. *15*, 5001–5013.

Iida, H., Yagawa, Y., and Anraku, Y. (1990). Essential role for induced Ca2+ influx followed by [Ca2+]i rise in maintaining viability of yeast cells late in the mating pheromone response pathway. A study of [Ca2+]i in single Saccharomyces cerevisiae cells with imaging of fura-2. J. Biol. Chem. *265*, 13391–13399.

Iida, H., Nakamura, H., Ono, T., Okumura, M.S., and Anraku, Y. (1994). MID1, a novel Saccharomyces cerevisiae gene encoding a plasma membrane protein, is required for Ca2+ influx and mating. Mol. Cell. Biol. *14*, 8259–8271.

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

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. U. S. A. *98*, 4569–4574.

Izawa, S., Inoue, Y., and Kimura, A. (1996). Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic Saccharomyces cerevisiae. Biochem. J. *320 (Pt 1*, 61–67.

Jiang, R., and Carlson, M. (1996). Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. Genes Dev. *10*, 3105–3115.

Jiang, R., and Carlson, M. (1997). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. Mol. Cell. Biol. *17*, 2099–2106.

Kafadar, K.A., and Cyert, M.S. (2004). Integration of stress responses: modulation of calcineurin signaling in Saccharomyces cerevisiae by protein kinase A. Eukaryot.Cell *3*, 1147–1153.

Kafadar, K.A., Zhu, H., Snyder, M., and Cyert, M.S. (2003). Negative regulation of calcineurin signaling by Hrr25p, a yeast homolog of casein kinase I. Genes Dev. *17*, 2698–2708.

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E.K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85 1 508 508. Science (80-.). *263*, 1153–1156.

Kaffman, A., Rank, N.M., and O'Shea, E.K. (1998a). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev. *12*, 2673–2683.

Kaffman, A., Rank, N.M., O'Neill, E.M., Huang, L.S., and O'Shea, E.K. (1998b). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus 2 513 513. Nature *396*, 482–486.

Kamada, Y., Jung, U.S., Piotrowski, J., and Levin, D.E. (1995). The protein kinase C-activated MAP kinase pathway of Saccharomyces cerevisiae mediates a novel aspect of the heat shock response. Genes Dev. *9*, 1559–1571.

Kaneko, Y., Tamai, Y., Toh-e, A., and Oshima, Y. (1985). Transcriptional and post-transcriptional control of PHO8 expression by PHO regulatory genes in Saccharomyces cerevisiae. Mol. Cell. Biol. *5*, 248–252.

Kanzaki, M., Nagasawa, M., Kojima, I., Sato, C., Naruse, K., Sokabe, M., and Iida, H. (1999). Molecular identification of a eukaryotic, stretch-activated nonselective cation channel. Science *285*, 882–886.

Ketela, T., Green, R., and Bussey, H. (1999). Saccharomyces cerevisiae mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J. Bacteriol. *181*, 3330–3340.

Ko, C.H., Buckley, A.M., and Gaber, R.F. (1990). TRK2 is required for low affinity K+ transport in Saccharomyces cerevisiae. Genetics *125*, 305–312.

Komeili, A., and O'Shea, E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science *284*, 977–980.

Korber, P., and Barbaric, S. (2014). The yeast PHO5 promoter: from single locus to systems biology of a paradigm for gene regulation through chromatin. Nucleic Acids Res. *42*, 10888–10902.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W., Donaton, M.C., Van Dijck, P., Winderickx, J., de Winde, J.H., and Thevelein, J.M. (1999). A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol. Microbiol. *32*, 1002–1012.

Kübler, E., Mösch, H.U., Rupp, S., and Lisanti, M.P. (1997). Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in Saccharomyces cerevisiae via a cAMP-dependent mechanism. J. Biol. Chem. *272*, 20321–20323.

Kuchin, S., Vyas, V.K., and Carlson, M. (2002). Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth, and diploid pseudohyphal differentiation. Mol. Cell. Biol. *22*, 3994–4000.

Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J. *16*, 1710–1720.

Kullas, A.L., Martin, S.J., and Davis, D. (2007). Adaptation to environmental pH: integrating the Rim101 and calcineurin signal transduction pathways. Mol. Microbiol. *66*, 858–871.

Kuret, J., Johnson, K.E., Nicolette, C., and Zoller, M.J. (1988). Mutagenesis of the regulatory subunit of yeast cAMP-dependent protein kinase. Isolation of site-directed mutants with altered binding affinity for catalytic subunit. J. Biol. Chem. *263*, 9149–9154.

Lamb, T.M., and Mitchell, A.P. (2003). The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes NRG1 and SMP1 in Saccharomyces cerevisiae. Mol. Cell. Biol. *23*, 677–686.

Lamb, T.M., Xu, W., Diamond, A., and Mitchell, A.P. (2001). Alkaline response genes of Saccharomyces cerevisiae and their relationship to the RIM101 pathway. J. Biol. Chem. *276*, 1850–1856.

Lazard, M., Blanquet, S., Fisicaro, P., Labarraque, G., and Plateau, P. (2010). Uptake of selenite by Saccharomyces cerevisiae involves the high and low affinity orthophosphate transporters. J. Biol. Chem. *285*, 32029–32037.

Lee, K.S., and Levin, D.E. (1992). Dominant mutations in a gene encoding a putative protein kinase (BCK1) bypass the requirement for a Saccharomyces cerevisiae protein kinase C homolog. Mol. Cell. Biol. *12*, 172–182.

Lee, K.S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D.E. (1993). A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. *13*, 3067–3075.

Lee, P., Cho, B.-R., Joo, H.-S., and Hahn, J.-S. (2008a). Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Mol. Microbiol. *70*, 882–895.

Lee, Y.-S., Mulugu, S., York, J.D., and O'Shea, E.K. (2007). Regulation of a cyclin-CDK-CDK inhibitor complex by inositol pyrophosphates. Science *316*, 109–112.

Lee, Y.-S., Huang, K., Quiocho, F.A., and O'Shea, E.K. (2008b). Molecular basis of cyclin-CDK-CKI regulation by reversible binding of an inositol pyrophosphate. Nat. Chem. Biol. *4*, 25–32.

Lesage, P., Yang, X., and Carlson, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol. Cell. Biol. *16*, 1921–1928.

Li, W., and Mitchell, A.P. (1997). Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. Genetics *145*, 63–73.

Li, M., Martin, S.J., Bruno, V.M., Mitchell, A.P., and Davis, D.A. (2004). Candida albicans Rim13p, a protease required for Rim101p processing at acidic and alkaline pHs. Eukaryot. Cell *3*, 741–751.

Lim, M.K., Siew, W.L., Zhao, J., Tay, Y.C., Ang, E., and Lehming, N. (2011). Galactose induction of the GAL1 gene requires conditional degradation of the Mig2 repressor. Biochem. J. *435*, 641–649.

Lin, S.S., Manchester, J.K., and Gordon, J.I. (2003). Sip2, an N-Myristoylated Subunit of Snf1 Kinase, Regulates Aging in Saccharomyces cerevisiae by Affecting Cellular Histone Kinase Activity, Recombination at rDNA Loci, and Silencing. J. Biol. Chem. *278*, 13390–13397.

Lo, H.J., Köhler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G.R. (1997). Nonfilamentous C. albicans mutants are avirulent. Cell *90*, 939–949.

Lo, W.S., Duggan, L., Emre, N.C., Belotserkovskya, R., Lane, W.S., Shiekhattar, R., and Berger, S.L. (2001). Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. Science *293*, 1142–1146.

Longtine, M., III, A.M., and Demarini, D. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

Lubin, M., and Ennis, H.L. (1964). On the role of intracellular potassium in protein synthesis. Biochim. Biophys. Acta *80*, 614–631.

Ludin, K., Jiang, R., and Carlson, M. (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *95*, 6245–6250.

Lundh, F., Mouillon, J.-M., Samyn, D., Stadler, K., Popova, Y., Lagerstedt, J.O., Thevelein, J.M., and Persson, B.L. (2009). Molecular mechanisms controlling phosphate-induced downregulation of the yeast Pho84 phosphate transporter. Biochemistry *48*, 4497–4505.

Lutfiyya, L.L., and Johnston, M. (1996). Two zinc-finger-containing repressors are responsible for glucose repression of SUC2 expression. Mol. Cell. Biol. *16*, 4790–4797.

Lutfiyya, L.L., Iyer, V.R., DeRisi, J., DeVit, M.J., Brown, P.O., and Johnston, M. (1998). Characterization of three related glucose repressors and genes they regulate in Saccharomyces cerevisiae. Genetics *150*, 1377–1391.

Madden, K., Sheu, Y.J., Baetz, K., Andrews, B., and Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. Science *275*, 1781–1784.

Marion, R.M., Regev, A., Segal, E., Barash, Y., Koller, D., Friedman, N., and O'Shea, E.K. (2004). Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. Proc. Natl. Acad. Sci. U. S. A. *101*, 14315–14322.

Márquez, J.A., and Serrano, R. (1996). Multiple transduction pathways regulate the sodium-extrusion gene PMR2/ENA1 during salt stress in yeast. FEBS Lett. *382*, 89–92.

Martin, D.C., Kim, H., Mackin, N.A., Maldonado-Báez, L., Evangelista, C.C., Beaudry, V.G., Dudgeon, D.D., Naiman, D.Q., Erdman, S.E., and Cunningham, K.W. (2011). New regulators of a high affinity Ca2+ influx system revealed through a genome-wide screen in yeast. J. Biol. Chem. *286*, 10744–10754.

Martinez, P., and Persson, B.L. (1998). Identification, cloning and characterization of a derepressible Na+-coupled phosphate transporter in Saccharomyces cerevisiae. Mol. Gen. Genet. MGG *258*, 628–638.

Martinez, P., Zvyagilskaya, R., Allard, P., and Persson, B.L. (1998). Physiological regulation of the derepressible phosphate transporter in Saccharomyces cerevisiae. J. Bacteriol. *180*, 2253–2256.

Martínez-Muñoz, G.A., and Kane, P. (2008). Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J. Biol. Chem. *283*, 20309–20319.

Martínez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. *15*, 2227–2235.

Matheos, D.P., Kingsbury, T.J., Ahsan, U.S., and Cunningham, K.W. (1997). Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae. Genes Dev. *11*, 3445–3458.

Matsumoto, T.K., Ellsmore, A.J., Cessna, S.G., Low, P.S., Pardo, J.M., Bressan, R.A., and Hasegawa, P.M. (2002). An osmotically induced cytosolic Ca2+ transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of Saccharomyces cerevisiae. J. Biol. Chem. *277*, 33075–33080.

Mayer, F.V., Heath, R., Underwood, E., Sanders, M.J., Carmena, D., McCartney, R.R., Leiper, F.C., Xiao, B., Jing, C., Walker, P.A., et al. (2011). ADP Regulates SNF1, the Saccharomyces cerevisiae Homolog of AMP-Activated Protein Kinase. Cell Metab. *14*, 707–714.

Mayordomo, I., Estruch, F., and Sanz, P. (2002). Convergence of the Target of Rapamycin and the Snf1 Protein Kinase Pathways in the Regulation of the Subcellular Localization of Msn2, a Transcriptional Activator of STRE (Stress Response Element)-regulated Genes. J. Biol. Chem. *277*, 35650–35656.

McCartney, R.R., and Schmidt, M.C. (2001). Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J. Biol. Chem. *276*, 36460–36466.

McCartney, R.R., Rubenstein, E.M., and Schmidt, M.C. (2005). Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. Curr. Genet. *47*, 335–344.

Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R., and de Larrinoa, I.F. (1998). Yeast putative transcription factors involved in salt tolerance. FEBS Lett. *425*, 323–328.

Mendizabal, I., Pascual-Ahuir, A., Serrano, R., and de Larrinoa, I.F. (2001). Promoter sequences regulated by the calcineurin-activated transcription factor Crz1 in the yeast ENA1 gene. Mol.Genet.Genomics *265*, 801–811.

Mendoza, I., Rubio, F., Rodriguez-Navarro, A., and Pardo, J.M. (1994). The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. J. Biol. Chem. *269*, 8792–8796.

Menoyo, S., Ricco, N., Bru, S., Hernández-Ortega, S., Escoté, X., Aldea, M., and Clotet, J. (2013). Phosphate-activated cyclin-dependent kinase stabilizes G1 cyclin to trigger cell cycle entry. Mol. Cell. Biol. *33*, 1273–1284.

Mingot, J.M., Espeso, E.A., Díez, E., and Peñalva, M.A. (2001). Ambient pH signaling regulates nuclear localization of the Aspergillus nidulans PacC transcription factor. Mol. Cell. Biol. *21*, 1688–1699.

Mira, N.P., Lourenço, A.B., Fernandes, A.R., Becker, J.D., and Sá-Correia, I. (2009). The RIM101 pathway has a role in Saccharomyces cerevisiae adaptive response and resistance to propionic acid and other weak acids. FEMS Yeast Res. *9*, 202–216.

Mitchelhill, K.I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L.A., and Kemp, B.E. (1994). Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase. J. Biol. Chem. *269*, 2361–2364.

Mizunuma, M., Hirata, D., Miyahara, K., Tsuchiya, E., and Miyakawa, T. (1998). Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. Nature *392*, 303–306.

Mizunuma, M., Hirata, D., Miyaoka, R., and Miyakawa, T. (2001). GSK-3 kinase Mck1 and calcineurin coordinately mediate Hsl1 down-regulation by Ca2+ in budding yeast. EMBO J. *20*, 1074–1085.

Moir, R.D., Lee, J., Haeusler, R.A., Desai, N., Engelke, D.R., and Willis, I.M. (2006). Protein kinase A regulates RNA polymerase III transcription through the nuclear localization of Maf1. Proc. Natl. Acad. Sci. U. S. A. *103*, 15044–15049.

Morgan, B.A., Banks, G.R., Toone, W.M., Raitt, D., Kuge, S., and Johnston, L.H. (1997). The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast Saccharomyces cerevisiae. EMBO J. *16*, 1035–1044.

Muller, E.M., Mackin, N.A., Erdman, S.E., and Cunningham, K.W. (2003). Fig1p facilitates Ca2+ influx and cell fusion during mating of Saccharomyces cerevisiae. J. Biol. Chem. *278*, 38461–38469.

Müller, O., Neumann, H., Bayer, M.J., and Mayer, A. (2003). Role of the Vtc proteins in V-ATPase stability and membrane trafficking. J. Cell Sci. *116*, 1107–1115.

Mulugu, S., Bai, W., Fridy, P.C., Bastidas, R.J., Otto, J.C., Dollins, D.E., Haystead, T.A., Ribeiro, A.A., and York, J.D. (2007). A Conserved Family of Enzymes That Phosphorylate Inositol Hexakisphosphate. Science (80-.). *316*, 106–109.

Murguia, J.R., Belles, J.M., and Serrano, R. (1995). A salt-sensitive 3'(2'),5'-bisphosphate nucleotidase involved in sulfate activation. Science (80-.). *267*, 232–234.

Musladin, S., Krietenstein, N., Korber, P., and Barbaric, S. (2014). The RSC chromatin remodeling complex has a crucial role in the complete remodeler set for yeast PHO5 promoter opening. Nucleic Acids Res. *42*, 4270–4282.

De Nadal, E., Casadomé, L., and Posas, F. (2003). Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. Mol. Cell. Biol. *23*, 229–237.

Nakamura, T., Liu, Y., Hirata, D., Namba, H., Harada, S., Hirokawa, T., and Miyakawa, T. (1993). Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. *12*, 4063–4071.

Navarrete, C., Petrezselyova, S., Barreto, L., Martinez, J.L., Zahradka, J., Arino, J., Sychrova, H., and Ramos, J. (2010). Lack of main K+ uptake systems in Saccharomyces cerevisiae cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions. FEMS Yeast Res. *10*, 508–517.

Nishizawa, M., Komai, T., Katou, Y., Shirahige, K., Ito, T., and Toh-e, A. (2008). Nutrient-regulated antisense and intragenic RNAs modulate a signal transduction pathway in yeast. PLoS Biol. *6*, 2817–2830.

Nishizawa, M., Tanigawa, M., Hayashi, M., Maeda, T., Yazaki, Y., Saeki, Y., and Toh-e, A. (2010). Pho85 kinase, a cyclin-dependent kinase, regulates nuclear accumulation of the Rim101 transcription factor in the stress response of Saccharomyces cerevisiae. Eukaryot. Cell *9*, 943–951.

Nobile, C.J., Solis, N., Myers, C.L., Fay, A.J., Deneault, J.-S., Nantel, A., Mitchell, A.P., and Filler, S.G. (2008). Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell. Microbiol. *10*, 2180–2196.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. EMBO J. *14*, 5931–5938.

O'Donnell, A.F., McCartney, R.R., Chandrashekarappa, D.G., Zhang, B.B., Thorner, J., and Schmidt, M.C. (2014). 2-Deoxyglucose impairs yeast growth by stimulating Snf1-regulated and α-arrestin-mediated trafficking of hexose transporters 1 and 3 in Saccharomyces cerevisiae. Mol. Cell. Biol. *35*, 939–955.

O'Neill, E.M., Kaffman, A., Jolly, E.R., and O'Shea, E.K. (1996). Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science *271*, 209–212.

Obara, K., and Kihara, A. (2014). Signaling events of the Rim101 pathway occur at the plasma membrane in a ubiquitination-dependent manner. Mol. Cell. Biol. *34*, 3525–3534.

Obara, K., Yamamoto, H., and Kihara, A. (2012). Membrane protein Rim21 plays a central role in sensing ambient pH in Saccharomyces cerevisiae. J. Biol. Chem. *287*, 38473–38481.

Ogawa, N., and Oshima, Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in Saccharomyces cerevisiae. Mol. Cell. Biol. *10*, 2224–2236.

Ogawa, N., DeRisi, J., and Brown, P.O. (2000). New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol.Biol.Cell *11*, 4309–4321.

Orejas, M., Espeso, E.A., Tilburn, J., Sarkar, S., Arst, H.N., and Penalva, M.A. (1995). Activation of the Aspergillus PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. *9*, 1622–1632.

Orij, R., Urbanus, M.L., Vizeacoumar, F.J., Giaever, G., Boone, C., Nislow, C., Brul, S., and Smits, G.J. (2012). Genome-wide analysis of intracellular pH reveals quantitative control of cell division rate by pHc in Saccharomyces cerevisiae. Genome Biol. *13*, R80.

Orlova, M., Kanter, E., Krakovich, D., and Kuchin, S. (2006). Nitrogen availability and TOR regulate the Snf1 protein kinase in Saccharomyces cerevisiae. Eukaryot. Cell *5*, 1831–1837.

Orlova, M., Barrett, L., and Kuchin, S. (2008). Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. Yeast *25*, 745–754.

Ostling, J., and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. *252*, 162–168.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y., and Takai, Y. (1996). Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in Saccharomyces cerevisiae. EMBO J. *15*, 2196–2207.

Paidhungat, M., and Garrett, S. (1997). A homolog of mammalian, voltage-gated calcium channels mediates yeast pheromone-stimulated Ca2+ uptake and exacerbates the cdc1(Ts) growth defect. Mol. Cell. Biol. *17*, 6339–6347.

Palmer, C.P., Zhou, X.L., Lin, J., Loukin, S.H., Kung, C., and Saimi, Y. (2001). A TRP homolog in Saccharomyces cerevisiae forms an intracellular Ca(2+)-permeable channel in the yeast vacuolar membrane 1. Proc.Natl.Acad.Sci.U.S A *98*, 7801–7805.

Panek, A.C., de Araujo, P.S., Moura Neto, V., and Panek, A.D. (1987). Regulation of the trehalose-6-phosphate synthase complex in Saccharomyces. I. Interconversion of forms by phosphorylation. Curr. Genet. *11*, 459–465.

Papamichos-Chronakis, M., Gligoris, T., and Tzamarias, D. (2004). The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. EMBO Rep. *5*, 368–372.

Park, S.H., Koh, S.S., Chun, J.H., Hwang, H.J., and Kang, H.S. (1999). Nrg1 is a transcriptional repressor for glucose repression of STA1 gene expression in Saccharomyces cerevisiae. Mol. Cell. Biol. *19*, 2044–2050.

Pattison-Granberg, J., and Persson, B.L. (2000). Regulation of cation-coupled high-affinity phosphate uptake in the yeast Saccharomyces cerevisiae. J. Bacteriol. *182*, 5017–5019.

Peñas, M.M., Hervás-Aguilar, A., Múnera-Huertas, T., Reoyo, E., Peñalva, M.A., Arst, H.N., and Tilburn, J. (2007). Further characterization of the signaling proteolysis step in the Aspergillus nidulans pH signal transduction pathway. Eukaryot. Cell *6*, 960–970.

Pérez-Sampietro, M., and Herrero, E. (2014). The PacC-family protein Rim101 prevents selenite toxicity in Saccharomyces cerevisiae by controlling vacuolar acidification. Fungal Genet. Biol. *71*, 76–85.

Pérez-Sampietro, M., Casas, C., and Herrero, E. (2013). The AMPK family member Snf1 protects Saccharomyces cerevisiae cells upon glutathione oxidation. PLoS One *8*, e58283.

Persson, B.L., Petersson, J., Fristedt, U., Weinander, R., Berhe, A., and Pattison, J. (1999). Phosphate permeases of Saccharomyces cerevisiae: structure, function and regulation. Biochim. Biophys. Acta *1422*, 255–272.

Persson, B.L., Lagerstedt, J.O., Pratt, J.R., Pattison-Granberg, J., Lundh, K., Shokrollahzadeh, S., and Lundh, F. (2003). Regulation of phosphate acquisition in Saccharomyces cerevisiae. Curr. Genet. *43*, 225–244.

Philip, B., and Levin, D.E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. Mol. Cell. Biol. *21*, 271–280.

Piña, F.J., O'Donnell, A.F., Pagant, S., Piao, H.L., Miller, J.P., Fields, S., Miller, E.A., and Cyert, M.S. (2011). Hph1 and Hph2 are novel components of the Sec63/Sec62 posttranslational translocation complex that aid in vacuolar proton ATPase biogenesis. Eukaryot. Cell *10*, 63–71.

Pinson, B., Vaur, S., Sagot, I., Coulpier, F., Lemoine, S., and Daignan-Fornier, B. (2009). Metabolic intermediates selectively stimulate transcription factor interaction and modulate phosphate and purine pathways. Genes Dev. *23*, 1399–1407.

Pittman, J.K., Cheng, N.-H., Shigaki, T., Kunta, M., and Hirschi, K.D. (2004). Functional dependence on calcineurin by variants of the Saccharomyces cerevisiae vacuolar Ca2+/H+ exchanger Vcx1p. Mol. Microbiol. *54*, 1104–1116.

Platara, M., Ruiz, A., Serrano, R., Palomino, A., Moreno, F., and Ariño, J. (2006). The transcriptional response of the yeast Na(+)-ATPase ENA1 gene to alkaline stress involves three main signaling pathways. J. Biol. Chem. *281*, 36632–36642.

Polizotto, R.S., and Cyert, M.S. (2001). Calcineurin-dependent nuclear import of the transcription factor Crz1p requires Nmd5p. J.Cell Biol. *154*, 951–960.

Portales-Casamar, E., Thongjuea, S., Kwon, A.T., Arenillas, D., Zhao, X., Valen, E., Yusuf, D., Lenhard, B., Wasserman, W.W., and Sandelin, A. (2010). JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. Nucleic Acids Res. *38*, D105–10.

Portillo, F., Mulet, J.M., and Serrano, R. (2005). A role for the non-phosphorylated form of yeast Snf1: tolerance to toxic cations and activation of potassium transport. FEBS Lett. *579*, 512–516.

Pratt, J.R., Mouillon, J.M., Lagerstedt, J.O., Pattison-Granberg, J., Lundh, K.I., and Persson, B.L. (2004). Effects of methylphosphonate, a phosphate analogue, on the expression and degradation of the high-affinity phosphate transporter Pho84, in Saccharomyces cerevisiae. Biochemistry *43*, 14444–14453.

Prior, C., Potier, S., Souciet, J.L., and Sychrova, H. (1996). Characterization of the NHA1 gene encoding a Na+/H+-antiporter of the yeast Saccharomyces cerevisiae. FEBS Lett. *387*, 89–93.

Proft, M., and Serrano, R. (1999). Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in Saccharomyces cerevisiae: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol. Cell. Biol. *19*, 537–546.

Proft, M., Pascual-Ahuir, A., de Nadal, E., Arino, J., Serrano, R., and Posas, F. (2001). Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J. *20*, 1123–1133.

Pujol-Carrion, N., Belli, G., Herrero, E., Nogues, A., and de la Torre-Ruiz, M.A. (2006). Glutaredoxins Grx3 and Grx4 regulate nuclear localisation of Aft1 and the oxidative stress response in Saccharomyces cerevisiae. J. Cell Sci. *119*, 4554–4564.

Qadota, H., Python, C.P., Inoue, S.B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D.E., and Ohya, Y. (1996). Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science *272*, 279–281.

Rajavel, M., Philip, B., Buehrer, B.M., Errede, B., and Levin, D.E. (1999). Mid2 is a putative sensor for cell integrity signaling in Saccharomyces cerevisiae. Mol. Cell. Biol. *19*, 3969–3976.

Ramon, A.M., Porta, A., and Fonzi, W.A. (1999). Effect of environmental pH on morphological development of Candida albicans is mediated via the PacC-related transcription factor encoded by PRR2. J. Bacteriol. *181*, 7524–7530.

Randez-Gil, F., Bojunga, N., Proft, M., and Entian, K.D. (1997). Glucose derepression of gluconeogenic enzymes in Saccharomyces cerevisiae correlates with phosphorylation of the gene activator Cat8p. Mol. Cell. Biol. *17*, 2502–2510.

Reinders, A., Bürckert, N., Boller, T., Wiemken, A., and De Virgilio, C. (1998). Saccharomyces cerevisiae cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. Genes Dev. *12*, 2943–2955.

Van der Rest, M.E., Kamminga, A.H., Nakano, A., Anraku, Y., Poolman, B., and Konings, W.N. (1995). The plasma membrane of Saccharomyces cerevisiae: structure, function, and biogenesis. Microbiol.Rev. *59*, 304–322.

Reynolds, A., Lundblad, V., Dorris, D., and Keaveney, M. (2001). Yeast vectors and assays for expression of cloned genes. Curr. Protoc. Mol. Biol. / Ed. by Frederick M. Ausubel ... [et Al *Chapter 13*, Unit13.6.

Rodríguez, C., Sanz, P., and Gancedo, C. (2003). New mutations of Saccharomyces cerevisiae that partially relieve both glucose and galactose repression activate the protein kinase Snf1. FEMS Yeast Res. *3*, 77–84.

Rodríguez-Galán, O., Galindo, A., Hervás-Aguilar, A., Arst, H.N., and Peñalva, M.A. (2009). Physiological involvement in pH signaling of Vps24-mediated recruitment of Aspergillus PalB cysteine protease to ESCRT-III. J. Biol. Chem. *284*, 4404–4412.

Rodriguez-Navarro, A., and Ramos, J. (1984). Dual system for potassium transport in Saccharomyces cerevisiae. J. Bacteriol. *159*, 940–945.

Rodríguez-Navarro, A., and Benito, B. (2010). Sodium or potassium efflux ATPase a fungal, bryophyte, and protozoal ATPase. Biochim. Biophys. Acta *1798*, 1841–1853.

Roomans, G.M., Blasco, F., and Borst-Pauwels, G.W. (1977). Cotransport of phosphate and sodium by yeast. Biochim. Biophys. Acta *467*, 65–71.

Ros, R., Montesinos, C., Rimon, A., Padan, E., and Serrano, R. (1998). Altered Na+ and Li+ homeostasis in Saccharomyces cerevisiae cells expressing the bacterial cation antiporter NhaA. J. Bacteriol. *180*, 3131–3136.

Rothfels, K., Tanny, J.C., Molnar, E., Friesen, H., Commisso, C., and Segall, J. (2005). Components of the ESCRT pathway, DFG16, and YGR122w are required for Rim101 to act as a corepressor with Nrg1 at the negative regulatory element of the DIT1 gene of Saccharomyces cerevisiae. Mol. Cell. Biol. *25*, 6772–6788.

Roy, J., and Cyert, M.S. (2009). Cracking the phosphatase code: docking interactions determine substrate specificity. Sci. Signal. *2*, re9.

Rubenstein, E.M., McCartney, R.R., Zhang, C., Shokat, K.M., Shirra, M.K., Arndt, K.M., and Schmidt, M.C. (2008). Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. J. Biol. Chem. *283*, 222–230.

Ruiz, A., Yenush, L., and Arino, J. (2003). Regulation of ENA1 Na(+)-ATPase gene expression by the Ppz1 protein phosphatase is mediated by the calcineurin pathway. Eukaryot.Cell *2*, 937–948.

Ruiz, A., González, A., García-Salcedo, R., Ramos, J., and Ariño, J. (2006). Role of protein phosphatases 2C on tolerance to lithium toxicity in the yeast Saccharomyces cerevisiae. Mol. Microbiol. *62*, 263–277.

Ruiz, A., Serrano, R., and Ariño, J. (2008). Direct regulation of genes involved in glucose utilization by the calcium/calcineurin pathway. J. Biol. Chem. *283*, 13923–13933.

Ruiz, A., Xu, X., and Carlson, M. (2011). Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. Proc. Natl. Acad. Sci. U. S. A. *108*, 6349–6354.

Ruiz, A., Xu, X., and Carlson, M. (2013). Ptc1 protein phosphatase 2C contributes to glucose regulation of SNF1/AMP-activated protein kinase (AMPK) in Saccharomyces cerevisiae. J. Biol. Chem. *288*, 31052–31058.

Rusnak, F., and Mertz, P. (2000). Calcineurin: form and function. Physiol. Rev. *80*, 1483–1521.

Rutherford, J.C., Jaron, S., and Winge, D.R. (2003). Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J. Biol. Chem. *278*, 27636–27643.

Sahli, S., Boulahfa, S., and Cornet, M. (2012). pH signaling inhibition in Candida albicans leads to enhanced activity and restore fungicidal effect of ergosterol synthesis inhibitors. 52nd Intersci. Conf. Anti- Microb. Agents Chemother., San Fr. CA.

Saiardi, A., Erdjument-Bromage, H., Snowman, A.M., Tempst, P., and Snyder, S.H. (1999). Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr. Biol. *9*, 1323–1326.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: A laboratoy manual (Cold Spring Harbor Laboratory Press, NY).

Sanglard, D., Ischer, F., Marchetti, O., Entenza, J., and Bille, J. (2003). Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. *48*, 959–976.

Sanz, P., Alms, G.R., Haystead, T.A., and Carlson, M. (2000). Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol. Cell. Biol. *20*, 1321–1328.

Schmidt, M.C., and McCartney, R.R. (2000). beta-subunits of Snf1 kinase are required for kinase function and substrate definition. EMBO J. *19*, 4936–4943.

Schneider, K.R., Smith, R.L., and O'Shea, E.K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. Science *266*, 122–126.

Schönholzer, F., Schweingruber, A.M., Trachsel, H., and Schweingruber, M.E. (1985). Intracellular maturation and secretion of acid phosphatase of Saccharomyces cerevisiae. Eur. J. Biochem. *147*, 273–279.

Sengottaiyan, P., Ruiz-Pavón, L., and Persson, B.L. (2013). Functional expression, purification and reconstitution of the recombinant phosphate transporter Pho89 of Saccharomyces cerevisiae. FEBS J. *280*, 965–975.

Serra-Cardona, A., Canadell, D., Ariño, J. (2015). Coordinate responses to alkaline pH stress in budding yeast. Microbial Cell (in press).

Serrano, R., Kielland-Brandt, M.C., and Fink, G.R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+- and Ca2+-ATPases. Nature *319*, 689–693.

Serrano, R., Ruiz, A., Bernal, D., Chambers, J.R., and Ariño, J. (2002). The transcriptional response to alkaline pH in Saccharomyces cerevisiae: evidence for calcium-mediated signalling. Mol. Microbiol. *46*, 1319–1333.

Serrano, R., Bernal, D., Simon, E., and Arino, J. (2004). Copper and iron are the limiting factors for growth of the yeast Saccharomyces cerevisiae in an alkaline environment. J. Biol. Chem. *279*, 19698–19704.

Serrano, R., Martin, H., Casamayor, A., and Arino, J. (2006). Signaling alkaline pH stress in the yeast Saccharomyces cerevisiae through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. J. Biol. Chem. *281*, 39785–39795.

Sethuraman, A., Rao, N.N., and Kornberg, A. (2001). The endopolyphosphatase gene: essential in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *98*, 8542–8547.

Shen, M.W.Y., Shah, D., Chen, W., and Da Silva, N. (2012). Enhanced arsenate uptake in Saccharomyces cerevisiae overexpressing the Pho84 phosphate transporter. Biotechnol. Prog. *28*, 654–661.

Shimizu, T., Toumoto, A., Ihara, K., Shimizu, M., Kyogoku, Y., Ogawa, N., Oshima, Y., and Hakoshima, T. (1997). Crystal structure of PHO4 bHLH domain-DNA complex: flanking base recognition. EMBO J. *16*, 4689–4697.

Shirahama, K., Yazaki, Y., Sakano, K., Wada, Y., and Ohsumi, Y. (1996). Vacuolar Function in the Phosphate Homeostasis of the Yeast Saccharomyces cerevisiae. Plant Cell Physiol. *37*, 1090–1093.

Shirra, M.K., Rogers, S.E., Alexander, D.E., and Arndt, K.M. (2005). The Snf1 protein kinase and Sit4 protein phosphatase have opposing functions in regulating TATA-binding protein association with the Saccharomyces cerevisiae INO1 promoter. Genetics *169*, 1957–1972.

Smith, F.C., Davies, S.P., Wilson, W.A., Carling, D., and Hardie, D.G. (1999). The SNF1 kinase complex from Saccharomyces cerevisiae phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. FEBS Lett. *453*, 219–223.

Sopko, R., Huang, D., Preston, N., Chua, G., Papp, B., Kafadar, K., Snyder, M., Oliver, S.G., Cyert, M., Hughes, T.R., et al. (2006). Mapping pathways and phenotypes by systematic gene overexpression. Mol. Cell *21*, 319–330.

Spielvogel, A., Findon, H., Arst, H.N., Araújo-Bazán, L., Hernández-Ortíz, P., Stahl, U., Meyer, V., and Espeso, E.A. (2008). Two zinc finger transcription factors, CrzA and SltA, are involved in cation homoeostasis and detoxification in Aspergillus nidulans. Biochem. J. *414*, 419–429.

Sreenivasan, A., Bishop, A.C., Shokat, K.M., and Kellogg, D.R. (2003). Specific inhibition of Elm1 kinase activity reveals functions required for early G1 events. Mol. Cell. Biol. *23*, 6327–6337.

Stathopoulos, A.M., and Cyert, M.S. (1997). Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. *11*, 3432–3444.

Steger, D.J., Haswell, E.S., Miller, A.L., Wente, S.R., and O'Shea, E.K. (2003). Regulation of chromatin remodeling by inositol polyphosphates. Science *299*, 114–116.

Su, S.S., and Mitchell, A.P. (1993a). Identification of functionally related genes that stimulate early meiotic gene expression in yeast. Genetics *133*, 67–77.

Su, S.S., and Mitchell, A.P. (1993b). Molecular characterization of the yeast meiotic regulatory gene RIM1. Nucleic Acids Res. *21*, 3789–3797.

Sudarsanam, P., Iyer, V.R., Brown, P.O., and Winston, F. (2000). Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. *97*, 3364–3369.

Szkotnicki, L., Crutchley, J.M., Zyla, T.R., Bardes, E.S.G., and Lew, D.J. (2008). The checkpoint kinase Hsl1p is activated by Elm1p-dependent phosphorylation. Mol. Biol. Cell *19*, 4675–4686.

Takatsume, Y., Ohdate, T., Maeta, K., Nomura, W., Izawa, S., and Inoue, Y. (2010). Calcineurin/Crz1 destabilizes Msn2 and Msn4 in the nucleus in response to Ca(2+) in Saccharomyces cerevisiae. Biochem. J. *427*, 275–287.

Tanaka, K., Matsumoto, K., and Toh-E, A. (1989). IRA1, an inhibitory regulator of the RAS-cyclic AMP pathway in Saccharomyces cerevisiae. Mol. Cell. Biol. *9*, 757–768.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990). IRA2, a second gene of Saccharomyces cerevisiae that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. Mol. Cell. Biol. *10*, 4303–4313.

Thomas, M.R., and O'Shea, E.K. (2005). An intracellular phosphate buffer filters transient fluctuations in extracellular phosphate levels. Proc. Natl. Acad. Sci. U. S. A. *102*, 9565–9570.

Thomas-Chollier, M., Defrance, M., Medina-Rivera, A., Sand, O., Herrmann, C., Thieffry, D., and van Helden, J. (2011). RSAT 2011: regulatory sequence analysis tools. Nucleic Acids Res. *39*, W86–91.

Thorsen, M., Perrone, G.G., Kristiansson, E., Traini, M., Ye, T., Dawes, I.W., Nerman, O., and Tamás, M.J. (2009). Genetic basis of arsenite and cadmium tolerance in Saccharomyces cerevisiae. BMC Genomics *10*, 105.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Peñalva, M.A., and Arst, H.N. (1995). The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. *14*, 779–790.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell *40*, 27–36.

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987a). Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277–287.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Hurwitz, M., Krebs, E.G., and Wigler, M. (1987b). Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in Saccharomyces cerevisiae. Mol. Cell. Biol. *7*, 1371–1377.

Toh-e, A., Tanaka, K., Uesono, Y., and Wickner, R.B. (1988). PHO85, a negative regulator of the PHO system, is a homolog of the protein kinase gene, CDC28, of Saccharomyces cerevisiae. Mol. Gen. Genet. *214*, 162–164.

Torres, L., Martin, H., Garcia-Saez, M.I., Arroyo, J., Molina, M., Sanchez, M., and Nombela, C. (1991). A protein kinase gene complements the lytic phenotype of Saccharomyces cerevisiae lyt2 mutants. Mol. Microbiol. *5*, 2845–2854.

Treitel, M.A., and Carlson, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein 1. Proc.Natl.Acad.Sci.U.S A *92*, 3132–3136.

Treitel, M.A., Kuchin, S., and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. Mol. Cell. Biol. *18*, 6273–6280.

Tréton, B., Blanchin-Roland, S., Lambert, M., Lépingle, A., and Gaillardin, C. (2000). Ambient pH signalling in ascomycetous yeasts involves homologues of the Aspergillus nidulans genes palF and palH. Mol. Gen. Genet. *263*, 505–513.

Tu, J., and Carlson, M. (1995). REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae 1. EMBO J. *14*, 5939–5946.

Tzamarias, D., and Struhl, K. (1995). Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters 1. Genes Dev. *9*, 821–831.

Ueda, Y., To-E, A., and Oshima, Y. (1975). Isolation and characterization of recessive, constitutive mutations for repressible acid phosphatase synthesis in Saccharomyces cerevisiae. J. Bacteriol. *122*, 911–922.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., et al. (2007). Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol.Cell *26*, 663–674.

Urech, K., Dürr, M., Boller, T., Wiemken, A., and Schwencke, J. (1978). Localization of polyphosphate in vacuoles of Saccharomyces cerevisiae. Arch. Microbiol. *116*, 275–278.

Venter, U., and Horz, W. (1989). The acid phosphatase genes PHO10 and PHO11 in S. cerevisiae are located at the telomeres of chromosomes VIII and I 1 491 491. Nucleic Acids Res. *17*, 1353–1369.

Verduyn, C., Postma, E., Scheffers, W.A., and Van Dijken, J.P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast *8*, 501–517.

Verna, J., Lodder, A., Lee, K., Vagts, A., and Ballester, R. (1997). A family of genes required for maintenance of cell wall integrity and for the stress response in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *94*, 13804–13809.

Viladevall, L., Serrano, R., Ruiz, A., Domenech, G., Giraldo, J., Barceló, A., and Ariño, J. (2004). Characterization of the calcium-mediated response to alkaline stress in Saccharomyces cerevisiae. J. Biol. Chem. *279*, 43614–43624.

Vincent, O., and Carlson, M. (1998). Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J. *17*, 7002–7008.

Vincent, O., and Carlson, M. (1999). Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. EMBO J. *18*, 6672–6681.

Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes Dev. *15*, 1104–1114.

Vyas, V.K., Kuchin, S., and Carlson, M. (2001). Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. Genetics *158*, 563–572.

Vyas, V.K., Berkey, C.D., Miyao, T., and Carlson, M. (2005). Repressors Nrg1 and Nrg2 regulate a set of stress-responsive genes in Saccharomyces cerevisiae. Eukaryot. Cell *4*, 1882–1891.

Wang, H., Liang, Y., Zhang, B., Zheng, W., Xing, L., and Li, M. (2011). Alkaline stress triggers an immediate calcium fluctuation in Candida albicans mediated by Rim101p and Crz1p transcription factors. FEMS Yeast Res. *11*, 430–439.

Wanke, V., Pedruzzi, I., Cameroni, E., Dubouloz, F., and De, V.C. (2005). Regulation of G0 entry by the Pho80-Pho85 cyclin-CDK complex. EMBO J. *24*, 4271–4278.

Watanabe, Y., Takaesu, G., Hagiwara, M., Irie, K., and Matsumoto, K. (1997). Characterization of a serum response factor-like protein in Saccharomyces cerevisiae, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol. Cell. Biol. *17*, 2615–2623.

Werner, A., and Kinne, R.K. (2001). Evolution of the Na-P(i) cotransport systems. Am. J. Physiol. Regul. Integr. Comp. Physiol. *280*, R301–12.

Westholm, J.O., Nordberg, N., Murén, E., Ameur, A., Komorowski, J., and Ronne, H. (2008). Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. BMC Genomics *9*, 601.

Wieland, J., Nitsche, A.M., Strayle, J., Steiner, H., and Rudolph, H.K. (1995). The PMR2 gene cluster encodes functionally distinct isoforms of a putative Na+ pump in the yeast plasma membrane. EMBO J. *14*, 3870–3882.

Wiesenberger, G., Steinleitner, K., Malli, R., Graier, W.F., Vormann, J., Schweyen, R.J., and Stadler, J.A. (2007). Mg2+ deprivation elicits rapid Ca2+ uptake and activates Ca2+/calcineurin signaling in Saccharomyces cerevisiae. Eukaryot. Cell *6*, 592–599.

Wilson, W.A., Hawley, S.A., and Hardie, D.G. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr. Biol. *6*, 1426–1434.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., et al. (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science (80-.). *285*, 901–906.

Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994). Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. J. Biol. Chem. *269*, 19509–19515.

Wurst, H., Shiba, T., and Kornberg, A. (1995). The gene for a major exopolyphosphatase of Saccharomyces cerevisiae. J. Bacteriol. *177*, 898–906.

Wykoff, D.D., and O'Shea, E.K. (2001). Phosphate transport and sensing in Saccharomyces cerevisiae. Genetics *159*, 1491–1499.

Wykoff, D.D., Rizvi, A.H., Raser, J.M., Margolin, B., and O'Shea, E.K. (2007). Positive feedback regulates switching of phosphate transporters in S. cerevisiae. Mol. Cell *27*, 1005–1013.

Xu, W., and Mitchell, A.P. (2001). Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. J. Bacteriol. *183*, 6917–6923.

Xu, W., Smith, F.J., Subaran, R., and Mitchell, A.P. (2004). Multivesicular body-ESCRT components function in pH response regulation in Saccharomyces cerevisiae and Candida albicans. Mol. Biol. Cell *15*, 5528–5537.

Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R.D. (1995). AFT1: a mediator of iron regulated transcriptional control in Saccharomyces cerevisiae. EMBO J. *14*, 1231–1239.

Ye, T., Elbing, K., and Hohmann, S. (2008). The pathway by which the yeast protein kinase Snf1p controls acquisition of sodium tolerance is different from that mediating glucose regulation. Microbiology *154*, 2814–2826.

Yokoyama, H., Mizunuma, M., Okamoto, M., Yamamoto, J., Hirata, D., and Miyakawa, T. (2006). Involvement of calcineurin-dependent degradation of Yap1p in Ca2+-induced G2 cell-cycle regulation in Saccharomyces cerevisiae. EMBO Rep. *7*, 519–524.

Yoshimoto, H., Saltsman, K., Gasch, A.P., Li, H.X., Ogawa, N., Botstein, D., Brown, P.O., and Cyert, M.S. (2002). Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in Saccharomyces cerevisiae. J. Biol. Chem. *277*, 31079–31088.

Young, E.T., Dombek, K.M., Tachibana, C., and Ideker, T. (2003). Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. J. Biol. Chem. *278*, 26146–26158.

Zakrzewska, A., Boorsma, A., Brul, S., Hellingwerf, K.J., and Klis, F.M. (2005). Transcriptional response of Saccharomyces cerevisiae to the plasma membrane-perturbing compound chitosan. Eukaryot.Cell *4*, 703–715.

Zhang, M., Galdieri, L., and Vancura, A. (2013). The yeast AMPK homolog SNF1 regulates acetyl coenzyme A homeostasis and histone acetylation. Mol. Cell. Biol. *33*, 4701–4717.

Zhao, C., Jung, U.S., Garrett-Engele, P., Roe, T., Cyert, M.S., and Levin, D.E. (1998). Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. Mol. Cell. Biol. *18*, 1013–1022.

Zhou, H., and Winston, F. (2001). NRG1 is required for glucose repression of the SUC2 and GAL genes of Saccharomyces cerevisiae 3. BMC.Genet. *2*, 5.

Zhou, X., and O'Shea, E.K. (2011). Integrated approaches reveal determinants of genome-wide binding and function of the transcription factor Pho4. Mol. Cell *42*, 826–836.

Zvyagilskaya, R.A., Lundh, F., Samyn, D., Pattison-Granberg, J., Mouillon, J.-M., Popova, Y., Thevelein, J.M., and Persson, B.L. (2008). Characterization of the Pho89 phosphate transporter by functional hyperexpression in Saccharomyces cerevisiae. FEMS Yeast Res. *8*, 685–696.

ANNEXES

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source or reference
DBY746	*MATα his3-1 leu2-3, 112 ura3-52 trp1-289*	D. Botstein
MAR15	DBY746 *cnb1::kanMX4*	Serrano *et al.* 2002
RSC2	DBY746 *crz1::kanMX4*	This work
MAR39	DBY746 *cnb1::TRP1 crz1::kanMX4*	Casado *et al.*, 2010
RSC4	DBY746 *pho4::LEU2*	Serrano et al. 2002
RSC10	DBY746 *snf1::LEU2*	Ruiz *et al.* 2006
RSC29	DBY746 *reg1::kanMX4*	This work
RSC30	DBY746 *reg1::kanMX4 snf1::LEU2*	This work
RSC31	DBY746 *rim101::kanMX4*	Serrano *et al.* 2002
RSC13	DBY746 *mig1::LEU2*	Platara *et al.* 2006
MP010	DBY746 *mig2::TRP1*	Platara *et al.* 2006
MP012	DBY746 *mig1::LEU2 mig2::TRP1*	Platara *et al.* 2006
MP014	DBY746 *snf1::LEU2 mig1::kanMX4*	Platara *et al.* 2006
MP015	DBY746 *snf1::LEU2 mig2::TRP1*	Platara *et al.* 2006
MP016	DBY746 *snf1::LEU2 mig1::kanMX4 mig2::TRP1*	Platara *et al.* 2006
MAR197	DBY746 *nrg1::nat1*	This work
MP018	DBY746 *nrg2::TRP1*	Platara *et al.* 2006
MAR205	DBY746 *nrg1::nat1 nrg2::TRP1*	This work
MAR206	DBY746 *rim101::kanMX4 nrg1::nat1*	Platara *et al.* 2006
MAR198	DBY746 *rim101::kanMX4 nrg2::TRP1*	Platara *et al.* 2006
MAR200	DBY746 *rim101::kanMX4 nrg1::nat1 nrg2::TRP1*	Platara *et al.* 2006
MP020	DBY746 *snf1::LEU2 nrg1::kanMX4*	Platara *et al.* 2006
MP021	DBY746 *snf1::LEU2 nrg2::kanMX4*	Platara *et al.* 2006
MP022	DBY746 *snf1::LEU2 nrg1::kanMX4 nrg2::TRP1*	Platara *et al.* 2006
MAR196	DBY746 *mig2::TRP1 nrg2::kanMX4*	This work
MAR204	DBY746 *mig2::TRP1 nrg2::kanMX4 nrg1::nat1*	This work
MAR199	DBY746 *mig1::LEU2 mig2::TRP1 nrg2::kanMX4 nrg1::nat1*	Platara *et al.* 2006
ASC07	DBY746 *PHO89-3xHA-HIS3MX6*	This work
ASC08	DBY746 *PHO89-3xHA-HIS3MX6 cnb1::kanMX4*	This work
ASC09	DBY746 *PHO89-3xHA-HIS3MX6 snf1::LEU2*	This work

ASC10	DBY746 *PHO89-3xHA-HIS3MX6 pho4::LEU2*	This work
ASC11	DBY746 *PHO89-3xHA-HIS3MX6 rim101::kanMX4*	This work
ASC13	DBY746 *pho89::kanMX4*	This work
ASC14	DBY746 *snf1::LEU2 pho4::kanMX4*	This work
ASC15	DBY746 *rim101::HIS3MX6 pho4::kanMX4*	This work
ASC17	DBY746 *ena1-ena4::LEU2 pho84::kanMX4*	This work
CNA1	DBY746 *pho84::kanMX4*	This work
REN66	DBY746 *ena1-ena4::LEU2*	Haro *et al.*, 1991
ASC18	DBY746 *ENA1-3xHA-HIS3MX6*	This work
ASC19	DBY746 *ena1-ena4::LEU2 pho89::kanMX4*	This work
RSC18	DBY746 *pho84::kanMX4 pho89::TRP1*	This work
SP048	DBY746 *MIG2-GFP-HIS3MX6*	This work
ASC24	DBY746 *reg1::kanMX4 MIG2-GFP-HIS3MX6*	This work
ASC24	DBY746 *reg1::kanMX4 PHO89-3xHA-HIS3MX6*	This work
ASC25	DBY746 *reg1::kanMX4 snf1::LEU2 PHO89-3xHA-HIS3MX6*	This work
ASC45	DBY746 *snf1::LEU2 mig1::LEU2 mig2::TRP1 PHO89-3xHA-HIS3MX6*	This work
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al.* 1998
SP002	BY4741 *MIG2-GFP-HIS3MX6*	This work
SP020	BY4741 *CRZ1-3xHA-kanMX6*	This work
SP041	BY4741 *MIG2-3xHA-kanMX6*	This work
SP044	BY4741 *MIG1-3xHA-kanMX6*	This work
SP048	BY4741 *MIG1-GFP-HIS3MX6*	This work
ASC26	BY4741 *MIG1-GFP-HIS3MX6 snf1::LEU2*	This work
ASC04	BY4741 *MIG2-GFP-HIS3MX6 snf1::LEU2*	This work

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence
PEX29-C-pTAC-dir	ATGGTATCATTTTGAATGCTCCTCGCTTTGGTGTGGAATACCAAATGACACGGATCCCCGGG TTAATTAA
PEX29-C-pTAC-rev	AGTTCTACCTAATGTTGTAAAACTGTATTCATAACCGAAACACATTATGAGAATTCGAGCTCG TTTAAAC
pex29_5'_CDREB_up	GATGTCACAGTAACTTTGGATCCTTCTATT
pex29_5'_CDREB_up	ATCCAAAGTTACTGTGACATCTAGTATTGGAC
PEX29_3_CDREB	GGGTGTGTGTATGTGCGATAGACCTTTTT
PEX29_3_CDREB	ATCGCACATACACACACCCTTGTGGTGAAGC
KL_PEX29_up1	TCTGGAAGGGTTCTCCAAAC
KL_PEX29_do1	GAAGCAATTGCACCTGTGAG
KL_ACT1_up2	TGCTGTCTTCCCATCTATCG
KL_ACT1_do2	ATTGAGCTTCATCACCAAC
MIG1-C-pTAC-dir	AAACATTACCACCCATAAGAAGTTTACCGTTGCCCTTCCCACACATGGACCGGATCCCCGGGT TAATTAA
MIG1-C-pTAC-rev	TTGTCTATTGTCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAGCGTAGAATTCGAGCTCG TTTAAAC
MIG2-C-pTAC-dir	TACGTAACCTACTGAAACAAATTGATGTTTTCAACGGTCCCAAAAGAGTTCGGATCCCCGGG TTAATTAA
MIG2-C-pTAC-rev	ATGACAGTTCGTTAGAGGAAAAATGGTGAGATAAAAAGGGGCCGTAAAGGGAATTCGAGCTC GTTTAAAC
CRZ1-C-pTAC-dir	TCACTCCCTTGTACGAAGAAGCCAGACAGGAGAAATCGGGACAAGAGAGTCGGATCCCCGGG TTAATTAA
CRZ1-C-pTAC-rev	TTATATAGAAAAAAAAAATTCCTATTCAAAGCTTAAAAAAACAAAAATAAGAATTCGAGCTC GTTTAAAC
5'flinc_KIMA01	AGATGGTGCCATTGGAAGATCTGCTTAATAAAGAAAATGGCACTGCGTACGCTGCAGGTCGA C
3'flinc_KIMA01	TTGGGATACTTGGTCAAGATGCGAACTGAGTTAGTTCCCATTTGGATCGATGAATTCGAGCT CG
18Sright	CAGCACGTGGGAGACAAATA
28Sleft	AGATGTCCACCCAGTAGACA
5'_CRZ1_flinc	TAGCAGTTGAGCTCCAGC

5'_CRZ1_fwd	GCTCTAGAACAGTGGCTCGTACC
5 'erg1_fwd	GGAGAACAAGAAGCCATAAAAG
3 'erg1_fwd	CGAGTTGTGCCTAAAACAAGCC
PHO84_prom_5'	GCGAATTCAGTATTACGCACGTTGG
Cter_PHO84_5'	CGGGATCCTGCTTCATGTTGAAGTTGAG
Pho89_prom_5'	CGGAATTCCTTACATCCGTTTAATTTC
Pho89_3'_SacI	CCGAGCTCTGTCATTTGGTATTCCACACC
PHO89_PHO84_1	CGGAATTCCATTATTTTGTTAGTGGG
pho89_3'_KanMX_2	ATACACATAGCCATGCCAGG
pho89_ext_5'	GGCTTTACATCAATTTGAC
pho89_ext_3'	TGTCATTTGGTATTCCACAC
HNA1-C-pYAC-rev	TACTACAATCCATACAGAAGTTAATATTGGTATTAAACAACGGATCCCCGGGTTAATTAA
HNA1-c-pYAC-rev	TGAATAAGGAAAAAGATAGGGAGCACTTAATAGGCCCTGCGAATTCGAGCTCGTTTAAAC
HNA1-pYAC-comp-Cter	AACTTTACAAGTGTGGAAAGAG
HNA1-pYAC-comp-Nter	ATAATCTCGTTTGCCATGC
pYAC_HNA_rev	GAACATCGTATGGGTAAAAGAT

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Coregulated Expression of the Na^+/Phosphate Pho89 Transporter and Ena1 Na^+-ATPase Allows Their Functional Coupling under High-pH Stress

Albert Serra-Cardona,[a] Silvia Petrezsélyová,[a]* David Canadell,[a] José Ramos,[b] Joaquín Ariño[a]

Institut de Biotecnologia i Biomedicina and Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain[a]; Departamento de Microbiología, Edificio Severo Ochoa, Universidad de Córdoba, Córdoba, Spain[b]

The yeast *Saccharomyces cerevisiae* has two main high-affinity inorganic phosphate (P_i) transporters, Pho84 and Pho89, that are functionally relevant at acidic/neutral pH and alkaline pH, respectively. Upon P_i starvation, *PHO84* and *PHO89* are induced by the activation of the *PHO* regulon by the binding of the Pho4 transcription factor to specific promoter sequences. We show that *PHO89* and *PHO84* are induced by alkalinization of the medium with different kinetics and that the network controlling Pho89 expression in response to alkaline pH differs from that of other members of the *PHO* regulon. In addition to Pho4, the *PHO89* promoter is regulated by the transcriptional activator Crz1 through the calcium-activated phosphatase calcineurin, and it is under the control of several repressors (Mig2, Nrg1, and Nrg2) coordinately regulated by the Snf1 protein kinase and the Rim101 transcription factor. This network mimics the one regulating expression of the Na^+-ATPase gene *ENA1*, encoding a major determinant for Na^+ detoxification. Our data highlight a scenario in which the activities of Pho89 and Ena1 are functionally coordinated to sustain growth in an alkaline environment.

Inorganic phosphate (P_i) is indispensable for the biosynthesis of key cellular components such as nucleic acids, nucleoproteins, and phospholipids and is involved in many metabolic and signaling pathways. Transport across the plasma membrane is the first step in the utilization of this essential nutrient. In the yeast *Saccharomyces cerevisiae*, the transport of P_i is mediated by several transporters that differ in their affinities for P_i. The low-affinity transport system (K_m, ~1 mM) is composed of the Pho87 and Pho90 H^+/P_i symporters, and it is sufficient for growth at normal external P_i concentrations (1–3). High-affinity P_i uptake (K_m, ~10 μM) is mediated by the plasma membrane Pho84 and Pho89 transporters (4–6). Pho84 cotransports phosphate with H^+, is mostly active at acidic pH, and is responsible for most of the high-affinity P_i uptake under normal growth conditions (7). Pho89 is a phosphate/cation symporter and works most efficiently under alkaline conditions, with an optimum pH of 9.5. Effective transport through Pho89 requires the existence of an alkali-metal cation gradient, with Na^+ being preferred over K^+ or Li^+ (5, 8).

In the presence of sufficient P_i in the external medium, the expression levels of both the *PHO84* and *PHO89* transporter-encoding genes are low. The expression levels of these genes are greatly increased in response to P_i starvation, which occurs through the activation of the *PHO* signaling pathway (9–11). This response involves the activation of the cyclin-dependent kinase inhibitor Pho81, which leads to the inhibition of the Pho85-Pho80 cyclin-dependent kinase complex. As a result, unphosphorylated Pho4 (a basic helix-loop-helix transcription factor) accumulates in the nucleus and binds to diverse phosphate-responsive gene promoters, including *PHO84* and *PHO89*, driving their transcriptional response. When extracellular P_i is not limiting, Pho81 is inactivated. As a consequence, the Pho85-Pho80 kinase complex becomes active and phosphorylates Pho4, resulting in its exclusion from the nucleus and thus triggering repression of the *PHO*-regulated genes (12–14).

In spite of the common regulatory traits mentioned above, evidence suggesting that *PHO84* and *PHO89* might respond differently to diverse external inputs or cellular conditions has been raised in the last few years. For instance, alkalinization of the medium results in the transcriptional activation of genes induced by phosphate starvation, including *PHO89* and *PHO84*. However, accumulation of *PHO89* mRNA occurs much faster than that of *PHO84* (15, 16). It is worth noting that in some cases, such as under alkaline stress, the involvement of signaling mechanisms other than the *PHO* pathway has been suggested (15, 16), pointing to the possible regulation of *PHO89* by the calcium-responsive calcineurin protein phosphatase pathway, which controls the activity of the Crz1/Tcn1 transcription factor (see reference 17 for a review).

Pho89 is a widely conserved protein with close homologs from bacteria to humans (18). On the basis of these precedents, we considered it necessary to explore in a systematic way the possible regulatory inputs governing the expression of the *PHO89* gene. Alkaline stress was employed as a transcriptional trigger because (i) it is a condition that results in powerful induction of *PHO89* expression and (ii) it has been shown to involve the modulation of a wide variety of signaling pathways in *S. cerevisiae* (15, 16, 19–23). Here we show that the expression patterns of Pho84 and Pho89 under high-pH stress are different. Contrary to Pho84, accumu-

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Address correspondence to Joaquín Ariño, Joaquin.Arino@uab.es.
* Present address: Silvia Petrezsélyová, Institute of Molecular Genetics of the AS CR, v.v.i., Prague, Czech Republic.
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lation of Pho89 is also regulated, aside from the Pho4 contribution, by the Crz1 transcription factor in response to calcineurin activation, mainly through a calcineurin-dependent response element (CDRE) located in the *PHO89* promoter at positions −273 to −267. In addition, we demonstrate that *PHO89* expression is also under the control of the Mig2 and Nrg1 repressors, in a way that implicates the Rim101 and Snf1 signaling pathways, and that this complex regulatory network is identical to the one described previously for alkaline induction of the Na^+-ATPase-encoding gene *ENA1* (24, 25). Ena1 is a major determinant of salt tolerance, and its mutation renders cells highly sensitive to sodium and lithium cations (26–28). Interestingly, *ena1* cells are also sensitive to alkaline pH even in the absence of high levels of external sodium (24, 29). Our work strongly suggests that the common signaling network that regulates Pho89 and Ena1 expression provides the molecular basis for a physiological interaction between the phosphate transporter and the sodium ATPase.

MATERIALS AND METHODS

Yeast strains and growth conditions. *S. cerevisiae* cells were grown at 28°C in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose) or, when carrying plasmids, in synthetic minimal medium lacking the appropriate selection requirements (30). Low-phosphate (low-P_i) medium was prepared from yeast nitrogen base (YNB)-based medium without amino acids and phosphate (catalog number CYN0803 [requested to also lack potassium salts]; Formedium Ltd., United Kingdom), which was made with 30 mM KCl and 100 μM potassium phosphate (except where otherwise stated). The equivalent high-phosphate (high-P_i) medium was made by adding 20 mM KCl and 10 mM potassium phosphate. In some experiments, YNB-based medium lacking phosphate and sodium was employed (catalog number CYN0810; Formedium Ltd.). For preparation of low-phosphate agar plates, purified agar (catalog number 1806.05; Conda) was employed.

Yeast strains used in the present study are described in Table 1. Strains RSC82, ONA1, and ASC13 were constructed by transformation of wild-type strain DBY746 with a disruption cassette amplified from the *crz1*::*kanMX4* (3.0-kbp), *pho84*::*kanMX4* (2.3-kbp), and *pho89*::*kanMX4* (2.1-kbp) mutants from the systematic disruption library in the BY4741 background (31). Strain RSC38 was made by transforming strain ONA1 with a *pho89*::*TRP1* deletion cassette obtained by amplification of the corresponding locus of strain PAM1 (7) with oligonucleotides pho89_orf_5′ and pho89_orf_3′. Strains ASC17 and ASC30 were constructed by disruption of the *PHO84* and *PHO89* genes with the cassettes described above in the RH16.6 (all four repeats at the *ENA* locus deleted) background. Strain RSC22, which carries the *rim101*::*HIS3* deletion in the DBY746 background, was made by short-flanking replacement with a 1.3-kbp cassette amplified from plasmid pFA6a-hisMx6 (32). Strain RSC89 was constructed by transforming wild-type strain DBY746 with a *reg1*::*kanMX4* cassette obtained by amplification from the BY4741 *reg1* deletion mutant with oligonucleotides 5′-reg1_disr and 3′-reg1_disr. Strain RSC90 was prepared by disruption of *SNF1* with a 3.9-kbp *snf1*::*LEU2* cassette from BamHI- and HindIII-digested plasmid pCC107 (33) in the RSC89 background. Strain ASC15 was made by deletion of the *PHO4* gene in strain RSC22 (see above) with a deletion cassette (1.6 kbp) obtained from the *pho4*::*kanMX4* BY4741 deletion mutant. Strain ASC14 was made similarly but by transforming strain RSC10 (34) with the *pho4*::*kanMX4* cassette.

Strain MAR193 was made by transforming *mig2* strain MP010 (24) with the *nrg2*::*kanMX4* cassette described previously in that same report. Strains MAR197, MAR205, and MAR204 were obtained by transformation of wild-type strain DBY746, strain MP018 (*nrg2*), and MAR193 (*mig2 nrg2*), respectively, with the 2.1-kbp *nrg1*::*nat1* cassette previously described (24). Strains ASC07, ASC08, ASC09, ASC10, and ASC11, carrying a version of *PHO89* encoding a C-terminally 3× hemagglutinin (3×HA)-tagged protein, were created by transformation of strains DBY476, MAR15 (*cnb1*), RSC10 (*snf1*), RSC4 (*pho4*), and RSC21 (*rim101*), respectively, with a cassette amplified with primers PHO89-C-pFA6-rev and PHO89-C-pFA6-dir from plasmid pFA6-3HA-His3MX6 (32). Strain SP011 contains a version of *MIG2* encoding a C-terminally 3×HA-tagged protein integrated into the same chromosomal locus and was

TABLE 1 Strains used in this work

Strain	Genotype	Source or reference
DBY746	*MATα his3-1 leu2-3,112 ura3-52 trp1-289*	D. Botstein
MAR15	DBY746 *cnb1*::*kanMX4*	15
RSC82	DBY746 *crz1*::*kanMX4*	This work
MAR89	DBY746 *cnb1*::*TRP1 crz1*::*kanMX4*	86
RSC4	DBY746 *pho4*::*LEU2*	15
RSC10	DBY746 *snf1*::*LEU2*	34
RSC89	DBY746 *reg1*::*kanMX4*	This work
RSC90	DBY746 *reg1*::*kanMX4 snf1*::*LEU2*	This work
RSC21	DBY746 *rim101*::*kanMX4*	15
RSC13	DBY746 *mig1*::*LEU2*	24
MP010	DBY746 *mig2*::*TRP1*	24
MP012	DBY746 *mig1*::*LEU2 mig2*::*TRP1*	24
MP014	DBY746 *snf1*::*LEU2 mig1*::*kanMX4*	24
MP015	DBY746 *snf1*::*LEU2 mig2*::*TRP1*	24
MP016	DBY746 *snf1*::*LEU2 mig1*::*kanMX4 mig2*::*TRP1*	24
MAR197	DBY746 *nrg1*::*nat1*	This work
MP018	DBY746 *nrg2*::*TRP1*	24
MAR205	DBY746 *nrg1*::*nat1 nrg2*::*TRP1*	This work
MAR206	DBY746 *rim101*::*kanMX4 nrg1*::*nat1*	24
MAR195	DBY746 *rim101*::*kanMX4 nrg2*::*TRP1*	24
MAR200	DBY746 *rim101*::*kanMX4 nrg1*::*nat1 nrg2*::*TRP1*	24
MP020	DBY746 *snf1*::*LEU2 nrg1*::*kanMX4*	24
MP021	DBY746 *snf1*::*LEU2 nrg2*::*kanMX4*	24
MP022	DBY746 *snf1*::*LEU2 nrg1*::*kanMX4 nrg2*::*TRP1*	24
MAR193	DBY746 *mig2*::*TRP1 nrg2*::*kanMX4*	This work
MAR204	DBY746 *mig2*::*TRP1 nrg2*::*kanMX4 nrg1*::*nat1*	This work
MAR199	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg2*::*kanMX4 nrg1*::*nat1*	24
ASC07	DBY746 *PHO89*-3×HA-*HIS3*	This work
ASC08	DBY746 *PHO89*-3×HA-*HIS3 cnb1*::*kanMX4*	This work
ASC09	DBY746 *PHO89*-3×HA-*HIS3 snf1*::*LEU2*	This work
ASC10	DBY746 *PHO89*-3×HA-*HIS3 pho4*::*LEU2*	This work
ASC11	DBY746 *PHO89*-3×HA-*HIS3 rim101*::*kanMX4*	This work
ASC13	DBY746 *pho89*::*kanMX4*	This work
ASC14	DBY746 *snf1*::*LEU2 pho4*::*kanMX4*	This work
ASC15	DBY746 *rim101*::*His3Mx pho4*::*kanMX4*	This work
ASC17	DBY746 *ena1–ena4*::*LEU pho84*::*kanMX4*	This work
ONA1	DBY746 *pho84*::*kanMX4*	This work
RH16.6	DBY746 *ena1–ena4*::*LEU2*	29
ASC28	DBY746 *ENA1*-3×HA-*HIS3*	This work
ASC30	DBY746 *ena1–ena4*::*LEU2 pho89*::*kanMX4*	This work
RSC38	DBY746 *pho84*::*kanMX4 pho89*::*TRP1*	This work
SP048	DBY746 *MIG2-GFP-HIS3*	This work
ASC34	DBY746 *reg1*::*kanMX4 MIG2-GFP-HIS3*	This work
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	87
SP002	BY4741 *MIG2-GFP-HIS3*	This work
SP020	BY4741 *CRZ1*-3×HA-*kanMX6*	This work
SP011	BY4741 *MIG2*-3×HA-*kanMX6*	This work
SP014	BY4741 *MIG1*-3×HA-*kanMX6*	This work
SP018	BY4741 *MIG1-GFP-kanMX6*	This work
ASC03	BY4741 *MIG1*-3×HA-*kanMX6 snf1*::*LEU2*	This work
ASC04	BY4741 *MIG2*-3×HA-*kanMX6 snf1*::*LEU2*	This work

constructed by transforming wild-type BY4741 cells with a cassette made by amplification of plasmid pFA6-3HA-kanMX6 (32) using oligonucleotides MIG2-C-pFA6-dir and MIG2-C-pFA6-rev. The same template was employed to amplify cassettes to generate a chromosomally encoded C-terminally 3×HA-tagged version of the *MIG1* or *CRZ1* protein in the BY4741 background (strains SP014 and SP020). The oligonucleotide pairs used were MIG1-C-pFA6-dir/MIG1-C-pFA6-rev (for SP014) and CRZ1-C-pFA6-dir/CRZ1-C-pFA6-rev (for SP020). Strain ASC28 was constructed by transformation of DBY746 cells with an ENA1-3×HA-His3 cassette generated by PCR with primers ENA1-C-pFA6-dir and ENA1-c-pFA6-rev and plasmid pFA6-3HA-His3MX6 (32) as the template. Because of the highly conserved sequence of the members of the *ENA* cluster, particular care was taken to ensure the selection of transformants carrying the tag specifically at the C terminus of the *ENA1* protein. To this end, positive clones were verified with two pairs of primers: ENA1-pFA6-comp-Cter and pFA6a_3HA_rev, which amplify a 0.2-kbp region irrespective of the member of the cluster receiving the tag, and ENA1-pFA6-comp-Nter and pFA6a_3HA_rev, which yield a 3.8-kb amplification fragment specific for *ENA1* integration. Strains ASC03 and ASC04 were obtained from strains SP014 and SP011, respectively, by transformation with the previously described *snf1*::*LEU2* cassette (34). Strains expressing Mig1-green fluorescent protein (GFP) (SP018) or Mig2-GFP (SP002, SP048, and ASC34) fusions were prepared by transforming either wild-type strain BY4741, DBY746, or RSC89 (*reg1*) with amplification fragments obtained by using plasmid pFA6-GFP(S65T)-kanMX6 (for Mig1) or pFA6-GFP(S65T)-HIS3MX6 (for Mig2) (32) and oligonucleotide pairs MIG1-C-pFA6-dir/MIG1-C-pFA6-rev and MIG2-C-pFA6-dir/MIG2-C-pFA6-rev, respectively. Transformants were verified by yeast colony PCR as well as by fluorescence microscopy. Oligonucleotides used in this work are described in Table S2 in the supplemental material.

Plasmids and recombinant DNA techniques. *Escherichia coli* DH5α cells were used as a plasmid DNA host and were grown at 37°C in LB (Luria-Bertani) broth supplemented, if necessary, with 100 μg/ml ampicillin. Recombinant DNA techniques and bacterial and yeast cell transformations were performed by using standard methods. Plasmid pPHO89-LacZ is a YEp357-based reported plasmid in which the region from positions −671 to +33 (relative to the initiating ATG codon) is translationally fused to the *lacZ* gene (15). Mutation of the upstream CDRE (CDRE1) in pPHO89-LacZ to generate PHO89^{CDRE1}-LacZ was accomplished by two-step PCR mutagenesis with specific oligonucleotides PHO89_5_CDRE1 and PHO89_3_CDRE1, so the native sequence GTG GCTG was changed to GTG*TA*TG (modified nucleotides are shown in italics). Similarly, PHO89^{CDRE2}-LacZ contained a mutated version of the downstream CDRE (CDRE2) and required oligonucleotides pho89_5′_CDRE2_m and pho89_3′_CDRE2_m, which promoted the change from CAGCCAC to CAG*TA*AC. Plasmid pMM15-PHO84, which expresses the *PHO84* protein from its native promoter with a 3×HA C-terminal tag, was made by PCR amplification of the relevant *PHO84* locus (nucleotides [nt] −598 to +1760 from the Met initiation codon) with oligonucleotides PHO84_prom_5′ and Clon_PHO84_3′ (carrying EcoRI- and BamHI-added sites, respectively) and cloning into these same sites of the centromeric plasmid (*URA3* marker). Similarly, plasmid pMM17-PHO89, expressing the *PHO89* protein C-terminally fused to the 3×HA epitope, was made by amplification of the *PHO89* locus (nt −671 to +1722) with oligonucleotides Pho89_prom_5′ and Pho89_3′_SacI and cloned into the EcoRI and SacI sites of plasmid pMM17 (centromeric, *LEU2* marker). Plasmids pMM15 and pMM17 were generous gifts from E. Herrero (Universitat de Lleida, Spain). Plasmid pKC201, containing *ENA1* sequences from nt −1385 to +35 (relative to the initiating Met) fused to *lacZ*, was described previously (35, 36). Plasmid pAMS366 drives the expression of the *lacZ* reporter gene from four copies in tandem with the CDRE present in the *FKS2* promoter (37).

Preparation of yeast extracts and immunoblot analysis. Yeast cells were subjected to high-pH, high-salt, and/or low-phosphate conditions as follows. For low-phosphate stress, 50 ml of cells was grown until the optical density at 660 nm (OD_{660}) reached 0.6 in high-P_i medium, and cells were collected by centrifugation (3 min at 1,228 × *g*), rinsed with low-P_i medium, and resuspended in 50 ml of low-P_i medium. For alkaline-stress experiments, 50 ml of cultures grown on YPD or YNB-based (low- or high-P_i) medium (pH 5.8) (OD_{660} = 0.6) was shifted to pH 8.0 by the addition of the appropriate volume of a 1 M KOH stock solution. For salt stress, the appropriate amount of solid NaCl was added to the cultures. For Pho89, Pho84, and Ena1 detection, 5 ml of cells was collected at the indicated times by rapid vacuum filtration through 0.45-μm Metricel GN-6 filters (Pall Corp.). Cells were quickly frozen in dry ice and stored at −80°C until use. Extracts were prepared for 10% SDS-PAGE (7% gels for Ena1) as previously described (8, 38). To detect phosphorylated Snf1 and the Snf1 protein, as well as the mobility shift of Mig1 and Mig2 upon alkaline-pH stress, cultures (5 to 10 ml) were made with 5.5% trichloroacetic acid and stored on ice for 15 min, and the pellet was collected by centrifugation (2 min at 4°C at 1,200 × *g*), washed twice with acetone, air dried, and stored at −80°C. For Mig1 and Mig2 mobility shifts, samples were processed as previously described (39, 40), except that when breaking the cells, EDTA was omitted from the urea buffer and each cell breakage round was extended to 45 s. Extracts were centrifuged for 10 min at 4°C in a microcentrifuge, the supernatant was recovered, and the concentration of proteins was determined by the Bradford method (Sigma Chemical Co.). For Snf1 detection, samples were resuspended in 150 μl of 10 mM Tris (pH 7.5)–1 mM EDTA buffer and then processed as described previously (41). Extracts were subjected to SDS-PAGE (8 or 10% polyacrylamide gels). Samples containing tagged Mig1 or Mig2 were treated with 10 units of calf alkaline phosphatase (Roche) for 90 min at 37°C, in the presence or in the absence of 50 mM EDTA.

In all cases, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). HA-tagged Pho89, Ena1, and Mig2 were detected by means of mouse monoclonal anti-HA antibody 12CA4 at a 1:1,000 dilution (Roche), whereas the HA.11 clone 16B12 monoclonal antibody (Covance) was employed for detection of HA-tagged Pho84 and Mig1. A 1:20,000 dilution of secondary anti-mouse IgG–horseradish peroxidase (GE Healthcare) was used. Phosphorylated Snf1 and Snf1 protein were monitored with anti-phospho-Thr172-AMPK (Cell Signaling Technology) and anti-His (GE Healthcare) antibodies, respectively. Actin was detected with the (I-19)-R rabbit polyclonal antibody (catalog number sc-1616-R; Santa Cruz Biotechnology) followed by ECL anti-rabbit IgG–horseradish peroxidase secondary antibodies (GE Healthcare). Immunoreactive proteins were visualized with the ECL Select or Prime (for Pho84-HA detection) kit (GE Healthcare).

Chromatin immunoprecipitation experiments. For chromatin immunoprecipitation (ChIP) experiments, strain SP020, encoding a *CRZ1*-3×HA version, was grown on YPD to an OD_{600} of 0.6 to 0.8 (~50 ml/time point). At time zero (t_0), the pH of the culture was increased to 8.0 by the addition of 1 M KOH, and growth resumed. Forty milliliters of cultures was taken at the appropriate times, and formaldehyde was added to a final concentration of 1% to cross-link proteins and DNA. From this point, the procedure was performed as described previously (40), with the following modifications. (i) Chromosomal DNA was fragmented by using Bioruptor Plus UCD-300 equipment (Diagenode) provided with a cooling system (4°C) for 45 cycles (high intensity; 30 s of sonication followed by a 60-s pause) to generate fragments ≤300 bp in length. (ii) The cleared lysate was precleared by the addition of ~20 μl of ChIP-grade protein G-Sepharose beads (catalog number 16-201; Millipore) to each sample, followed by incubation for 1 h with gentle rotation at 4°C. Samples were centrifuged at 220 × *g* for 1 min at 4°C, and the supernatant was transferred into screw-cap tubes. One microliter of anti-HA antibodies (catalog number ab9110; Abcam) was added to each precleared sample (except for the no-IP controls), and incubation continued overnight at 4°C. Fifty microliters to 100 μl of ChIP-grade protein G-Sepharose beads was added to each sample (including controls), and incubation continued for 1 h at 4°C until samples were centrifuged at 220 × *g* for 1 min at 4°C. (iii) Treatment with proteinase K was extended for 2 h at 37°C. Finally, the

eluted DNA was purified as described previously (40). For PCR assays, 40 ng of immunoprecipitated DNA was used together with oligonucleotides 18Right and 23Left. The procedures for ChIP-sequencing (ChIP-Seq) using these samples will be described elsewhere (not shown). Reads (FastQ files, 150-nt paired ends) were mapped with Bowtie 2 software (42). Mapped reads (1.9 million to 3.7 million/time point) were processed with IGV Tools (v 2.3) and quantified and represented with SeqMonk software (v 0.26.0).

Determination of intracellular sodium accumulation. Cells were grown on synthetic medium containing 30 mM HEPES (pH 5.5) in the presence of 5 mM Na^+, 10 mM K^+, and 10 mM P_i until the OD_{660} reached 0.3. Cultures were centrifuged at 1,200 × *g* for 5 min; washed with the same medium, in which the concentration of P_i was reduced to 0.2 mM; and resuspended in this low-P_i medium containing 15 mM K^+. Cells were grown for 60 min to induce the high-affinity phosphate transport system and then centrifuged and resuspended in the same medium, which was adjusted to pH 7.8 with KOH (which increases the concentration of K^+ to 18 mM). Five-milliliter aliquots were taken immediately after resuspension (t_0), and subsequent samples were taken at the desired times. Samples were rapidly filtered through 25-mm-diameter, 0.45-μm Metricel GN-6 filters (Pall Corp.) previously washed with 2.5 ml of a cold 20 mM $MgCl_2$ solution, and cells were washed with 10 ml of the $MgCl_2$ solution. Cells were recovered with 2 ml of this solution, filtered through a fresh filter, and subjected to a final wash. Filters were immersed in 10 ml of 10 mM $MgCl_2$–0.2 M HCl and gently shaken to resuspend the cells, and the intracellular sodium concentration was determined by atomic absorption spectrometry. Membranes used to filter medium without cells were processed in the same way and used for blank values. The number of cells in each aliquot was determined by measuring the OD_{660} at the moment of sampling, assuming that 1 OD unit equals 8×10^7 cells. A cellular volume of 49 pl was used for calculations, according to data described previously (43) and our own determinations for strain BY4741.

Confocal microscopy. Log-phase yeast cultures expressing GFP fusions growing in a low-fluorescence minimal medium lacking riboflavin and folic acid (44) were stained with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) to visualize nuclei. Cell suspensions were placed onto glass-bottom dishes and allowed to settle for a couple of minutes. Alkaline stress was initiated by gentle removal of the medium by using a pipette, placing the tip on the corner of the well, and the medium was then quickly replaced by low-fluorescence medium adjusted with 1 M KOH to pH 8.0. Images of living cells were taken before and after initiation of stress at different times with a confocal laser scanning microscope (Leica TCS SP2), using a 63× oil immersion objective. Images were analyzed by using LAS AF Lite (Leica Microsystems) and Wasabi (Hamamatsu Photonics Germany GmbH) imaging software.

Other techniques. A growth test in liquid cultures was performed as described previously (24). Growth on agar plates (dot tests) was carried out as described previously (28). *lacZ* reporter assays were performed essentially as described previously (15). For alkaline-pH stress, cells were collected after 90 min of shifting cells to pH 8.0. For NaCl stress, cells were exposed to 0.4 M (for 60 min) or to 0.8 M (for 90 min) NaCl.

PHO89 mRNA levels were determined by semiquantitative reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) with 15 ng of total RNA and oligonucleotides RT-PHO89-up1 and RT-PHO89-do1. For semiquantitative RT-PCR, the Illustra Ready-to-Go RT-PCR bead kit (GE Healthcare) was used. Reverse transcription was performed at 42°C for 30 min, and subsequent amplification was carried out for 23 cycles (1 min at 50°C and 72°C for annealing and extension conditions, respectively). qRT-PCR was performed with a CFX96 real-time system (Bio-Rad), using the QuantiTect SYBR green RT-PCR kit (Qiagen). Actin amplification was performed similarly but with oligonucleotides RT-ACT1-up2 and RT-ACT1-do2. Changes in *ENA1* mRNA levels were determined by DNA microarray experiments.

Relative Pho89 and Ena1 protein levels were calculated by the integration of immunoblot signals from YPD-grown cultures using GelAnalyzer software after background subtraction. Data for Pho89 were obtained from the integration of data from 3 experiments per time point, whereas data for Ena1 were obtained from a representative experiment.

In silico analysis of the *PHO89* promoter was carried out on the nucleotide sequence of the region comprising nucleotides −800 to −1 (from the initiating ATG) of *PHO89*, which was previously shown to contain high-pH response elements (15) and was analyzed with the matrix-scan algorithm available at the RSAT website (45), setting a *P* value threshold of 0.001. Matrices for transcription factor binding sequences were obtained from the JASPAR database (46).

RESULTS

Kinetics for Pho89 and Pho84 accumulation in response to low-phosphate and high-pH stress are different. Since high-pH stress triggers a response that mimics the situation of phosphate starvation, we considered it essential to compare the kinetics of Pho89 accumulation in cells subjected to these types of stress. To this end, a C-terminally HA-tagged version of Pho89 was engineered and introduced into its own chromosomal locus, and the resulting strain was grown in synthetic medium at standard pH (5.5) and then transferred to medium either adjusted to pH 8.0, containing low phosphate (100 μM), or with a combination of both circumstances. As shown in Fig. 1A, high pH resulted in a fast and transient accumulation of Pho89 (30 to 60 min), whereas the kinetic for Pho89 accumulation after transfer to low-phosphate medium was much slower, with detectable amounts of the transporter being found only 4 to 8 h after phosphate removal. As expected, the response to phosphate starvation was largely abolished in a *pho4* mutant. Exposure of cells to high pH and phosphate scarcity simultaneously yielded a profile of Pho89 expression that was a combination of that observed for each independent stress, that is, an early and transient peak followed by a late accumulation of the transporter (Fig. 1A).

In contrast, as shown in Fig. 1B, the temporal profile of Pho84 expression in response to high pH or phosphate starvation was essentially identical, peaking at ~180 min after initiation of the stress. In this case, the response was stronger under phosphate starvation conditions, but in both circumstances, it was fully abolished in the absence of the Pho4 transcription factor. Therefore, our results point out substantial differences in the expressions of Pho89 and Pho84 in response to high-pH stress: Pho89 expression occurs faster and does not mimic the kinetics observed upon phosphate starvation.

We also investigated whether the absence of one of the transporters could influence the expression of the other under P_i starvation or high-pH-stress conditions. We observed that while the *pho89* mutant displayed a Pho84 expression pattern identical to that of the wild-type strain (not shown), in *pho84* cells subjected to high-pH stress, expression of Pho89 was induced slightly earlier, and a second induction peak, similar to that observed upon P_i depletion, was found (see Fig. S1 in the supplemental material). This could be due to a higher-than-normal activation of Pho4 in Pho84-deficient cells, which would fit with the previously observed derepression of *PHO5* in this mutant (3).

Regulation of *PHO89* expression by the calcineurin/Crz1 pathway. Information retrieved from DNA microarray transcriptomic databases (15, 47, 48) and previous results from our laboratory (15, 16) suggested that *PHO89* expression may be influenced by the calcineurin pathway. To further investigate this process, we subjected wild-type DBY746 cells and their isogenic *cnb1* derivatives, lacking the regulatory subunit of the calcineurin

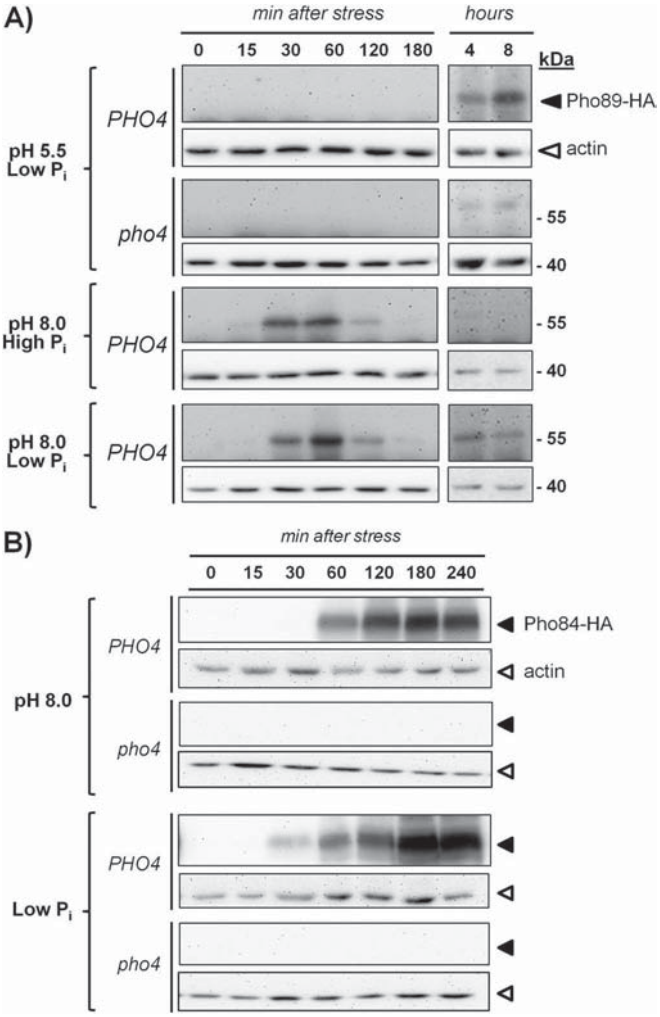

FIG 1 Comparative accumulations of Pho89 and Pho84 in response to phosphate starvation and high-pH stress. (A) Strains ASC07 (*PHO4 PHO89*-3×HA) and ASC10 (*pho4*::*LEU2 PHO89*-3×HA) were grown on synthetic high-P_i medium until the OD_{660} reached 0.6. Cells were collected and resuspended in synthetic medium lacking phosphate supplemented with 10 mM P_i (high P_i) or 100 μM P_i (low Pi) at pH 5.5 and/or pH 8.0 and adjusted with KOH. Growth was resumed, and samples were taken at the indicated times and processed for protein extract preparation as indicated in Materials and Methods. Equivalent amounts (30 μg of total protein) were subjected to SDS-PAGE (10% polyacrylamide gels) followed by immunoblotting using anti-HA antibodies to reveal HA-tagged Pho89 (dark triangles). Membranes were stripped and reprobed with antiactin antibodies (open triangles) for loading and transfer reference. (B) Wild-type strain DBY746 and its *pho4* derivative (RSC4) were transformed with centromeric plasmid pMM15-PHO84, which expresses the C-terminally HA-tagged version of Pho84. Cells were subjected to high-pH or low-phosphate stress and collected after the indicated periods, and the amount of Pho84 was assessed by using anti-HA antibodies. Correct loading and transfer were monitored with antiactin antibodies in the stripped membranes.

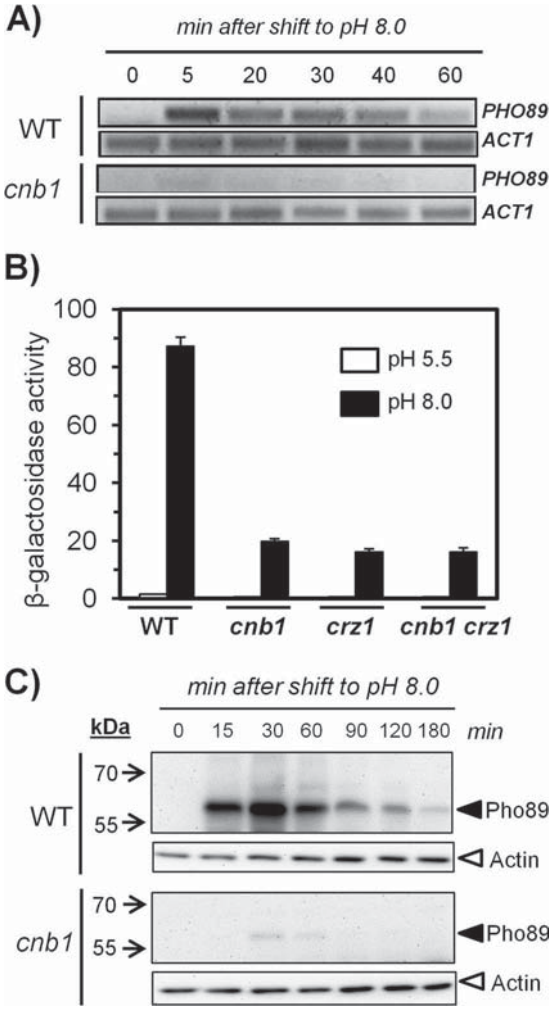

FIG 2 Expression of Pho89 in response to high-pH stress is controlled by the calcineurin/Crz1 pathway. (A) Wild-type (WT) DBY746 and MAR15 (*cnb1*) cells were collected at the indicated times after switching the medium to pH 8.0, and total RNA was prepared. Semiquantitative RT-PCR was carried out by using oligonucleotides specific for *PHO89* and *ACT1* (as a control), and the products were resolved in agarose gels and stained with GelRed (Biotium Inc.). (B) The indicated strains were transformed with the pPHO89-LacZ reporter, and exponentially growing cultures were exposed to pH 8.0 or maintained at pH 5.5 for 90 min. Cells were then collected, and β-galactosidase activity was measured as reported previously (15). Data are mean ± standard errors of the means from 12 determinations. (C) Cultures of strains ASC07 (*PHO89*-3×HA) and ASC08 (*cnb1 PHO89*-3×HA) were shifted to pH 8.0, and protein extracts were prepared. Samples (30 μg of protein) were resolved by SDS-PAGE and processed for immunoblotting as described in the legend to Fig. 1A.

phosphatase, to alkaline stress (pH 8.0) and prepared total RNA from cultures collected at different times. As shown in Fig. 2A, *PHO89* mRNA accumulated rapidly (5 min) in response to stress, and the mRNA levels declined only after 60 min, in agreement with the kinetics of accumulation of the Pho89 protein described above. This response was largely blocked in cells lacking calcineurin, thus confirming the participation of this pathway in the control of *PHO89* expression. Calcineurin activation results in dephosphorylation of the Crz1 transcription factor, its entry into the nucleus, and binding to specific sequences known as CDREs (calcineurin-dependent response elements). Therefore, a *lacZ* reporter gene fused to the *PHO89* promoter was introduced into wild-type, *cnb1*, *crz1*, and *cnb1 crz1* strains. As shown in Fig. 2B, the lack of the phosphatase, its downstream transcription factor, or both components resulted in a strong decrease, virtually identical in all cases, in the response of the promoter. As shown in Fig. 2C, when the production of the Pho89 protein was monitored in cells grown in rich medium (YPD), substantial accumulation was

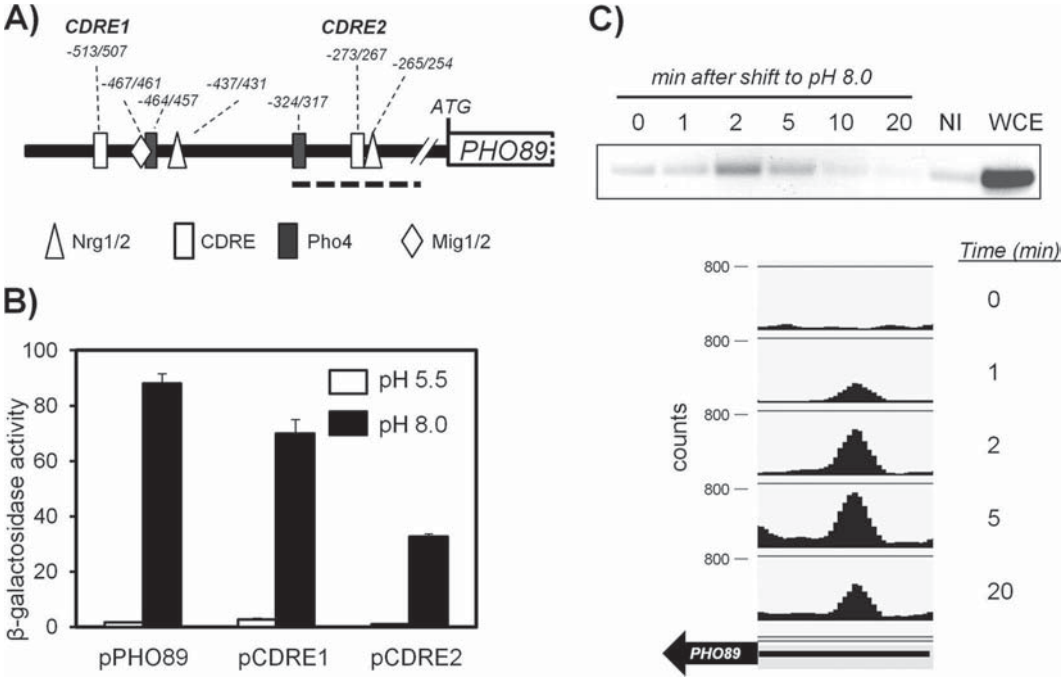

FIG 3 Functional characterization of two potential CDREs in the *PHO89* promoter. (A) Cartoon depicting the predicted regulatory sites in the *PHO89* upstream region (see the text for details). The bold discontinuous line spans the region amplified from ChIP samples shown in panel C. (B) Wild-type strain DBY746 was transformed with plasmid pPHO89-LacZ, pPHO89^{CDRE1}-LacZ, or pPHO89^{CDRE2}-LacZ, and cultures were subjected to pH 8.0 or maintained at pH 5.5. β-Galactosidase activity was determined as described in the legend to Fig. 2B. Data are means ± standard errors of the means from 12 determinations. (C, top) Chromatin-immunoprecipitated material from cells exposed to pH 8.0 for the indicated times was subjected to PCR amplification for the 143-nt region encompassing the CDRE2 consensus (discontinuous line in panel A). NI, sample lacking anti-HA antibodies; WCE, whole-cell extract. (Bottom) ChIP samples were subjected to massive sequencing. Mapped reads were quantified, normalized, and represented by using SeqMonk software. The thick arrow represents the *PHO89* open reading frame, and the thin line represents the 0.5-kbp upstream region (note that *PHO89* is on the Crick strand).

already observed after 15 min of exposure of the culture to high pH. This accumulation was strongly decreased in cells deficient in calcineurin activity. Therefore, our results indicate that calcineurin is involved in a major signaling pathway controlling the expression of the Pho89 transporter in response to high-pH stress. In contrast, deletion of *cnb1* did not alter the expression pattern of Pho84 under low-P_i or high-pH conditions (not shown), thus confirming the absence of calcineurin-mediated regulation of the *PHO84* promoter (15).

We then searched in the *PHO89* promoter for possible Crz1 binding regions as well as for sites for the Pho4 activator and other possible regulatory sites, including Nrg1/Nrg2 and Mig1/Mig2 (see Table S1 in the supplemental material). These two pairs of repressors were included in the search because they regulate the high-pH-responsive *ENA1* promoter (24). As shown in Fig. 3A, two possible consensus sites each for Crz1, Pho4, and Nrg1/2 plus a single putative Mig1/2 binding site were located in the region spanning nt −520 to −250 upstream of the initiating Met codon. No Msn2/Msn4 binding sites were identified in the analyzed region, suggesting that this promoter is not under general stress response regulation.

We first concentrated on the two putative CDRE binding sites, which are in fact identical sequences encoded in opposite DNA strands. The upstream sequence (CDRE1 [GTGGCTG]) was located at positions −513 to −507, whereas the second, with the sequence CAGCCAC, was found at positions −273 to −267 (CDRE2). We modified two nucleotides in each consensus sequence by site-directed mutagenesis to eliminate the conserved GCC core characteristic of the CDREs, thus changing CDRE1 to GTG*TA*TG and converting CDRE2 to CAG*TA*AC (modified nucleotides are shown in italics). We then tested the ability of these modified promoters to drive transcription of a *lacZ* reporter. As shown in Fig. 3B, mutation of CDRE1 resulted in a relatively minor decrease in β-galactosidase activity. In contrast, modification of CDRE2 strongly interfered with the function of the promoter, since the decrease in the response was almost as strong as the effect observed upon deletion of calcineurin or Crz1 (compare Fig. 3B with 2B). This suggests that CDRE2 is a major calcineurin-dependent regulatory site for *PHO89*. To further confirm the participation of this regulatory element in the control of *PHO89*, we performed chromatin immunoprecipitation experiments using strain SP020, which carries a C-terminally HA-tagged version of Crz1. As shown in Fig. 3C (top), the transcription factor is recruited very quickly to a region of the promoter that encompasses the CDRE2 sequence, showing a peak between 2 and 5 min after exposure to alkaline pH. This very fast response is in agreement with the rapid nuclear localization, upon a shift to alkaline pH, of a version of Crz1 fused to GFP (49; our unpublished data). In an independent experiment, ChIP samples were subjected to massive sequencing to obtain a genome-wide perspective of the binding of Crz1 to alkaline-pH-responsive promoters. Among the >100 promoters recognized by Crz1 upon high-pH stress (data not shown), time-dependent recruitment of Crz1 to the *PHO89* promoter was clearly detected (Fig. 3C, bottom).

Both Snf1 and Rim101 contribute to the induction of *PHO89* upon alkaline stress. Previous studies, including the characteriza-

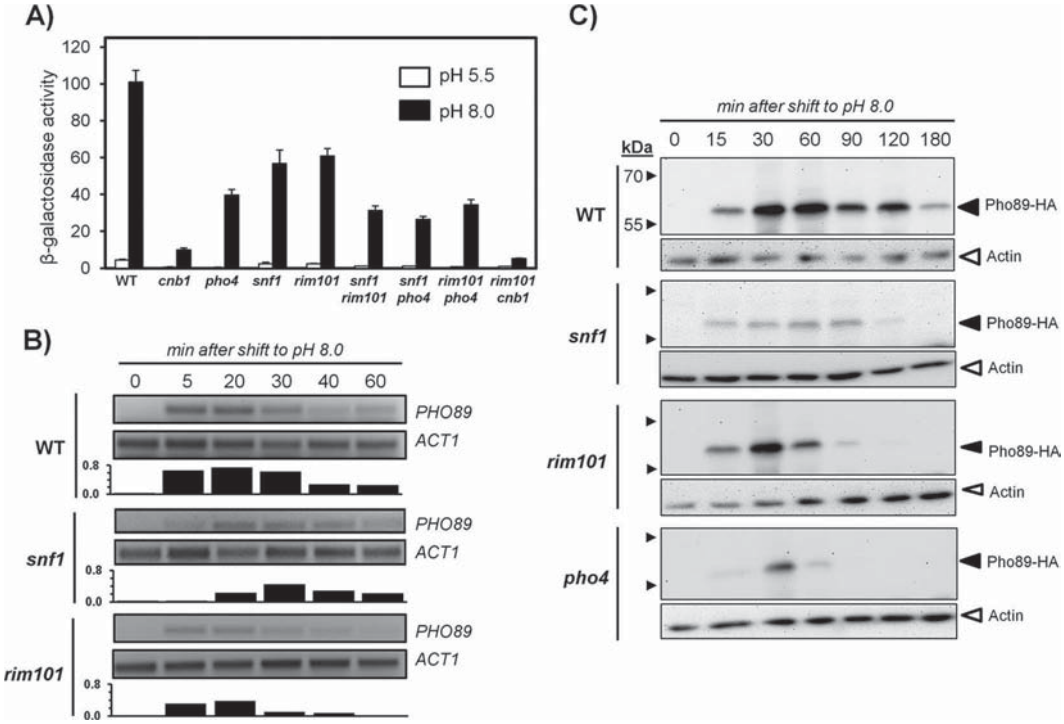

FIG 4 *PHO89* expression under conditions of high-pH stress is regulated by Snf1 and Rim101. (A) The indicated strains, transformed with the pPHO89-LacZ reporter, were exposed to pH 8.0 or maintained at pH 5.5, and β-galactosidase activity was determined. Data are means ± standard errors of the means from 10 to 15 experiments. (B) Wild-type DBY476, RSC10 (*snf1*), and RSC21 (*rim101*) cells were collected at the indicated times after switching the medium to pH 8.0, and total RNA was prepared. Semiquantitative RT-PCR was performed as described in the legend to Fig. 2A. Signals were integrated, and bars at the bottom of each panel denote the ratios between the *PHO89* and *ACT1* mRNAs for each time point and strain. (C) The above-mentioned strains plus strain RSC4 (*pho4*) were subjected to pH 8.0 for the indicated times and processed as described in the legend to Fig. 1A, and the amount of Pho89 was revealed by immunoblotting.

tion of the response of the *ENA1* promoter, showed that both Snf1 and Rim101 pathways could be activated by high pH (22, 24, 50). To investigate the possible impact of these signaling pathways on *PHO89* expression, we monitored the response of the *PHO89-lacZ* reporter in cells lacking Snf1 and Rim101 as well as in *pho4* mutants. As shown in Fig. 4A, the absence of Snf1 decreased the response of the reporter by ~40% compared to the wild type. This effect was similar in potency to the decrease observed upon deletion of *RIM101*, whereas a lack of Pho4 had a somewhat stronger impact. However, in all cases, the effect was less prominent than that observed in calcineurin-deficient cells. Deletion of *SNF1* in a *rim101* or a *pho4* background resulted in a further reduction of promoter activity. Whereas deletion of *RIM101* in the *pho4* background decreased the promoter response only marginally, the lack of Rim101 in a calcineurin-deficient strain further blocked the response of the *PHO89* promoter upon shifting the cells to pH 8.0. These results indicate that, in most cases, concurrent elimination of the above-mentioned components is additive, thus suggesting that they control independent signaling pathways acting on the *PHO89* promoter. The loss of response in *snf1* and *rim101* mutants was confirmed by monitoring *PHO89* mRNA levels by RT-PCR (Fig. 4B). Similarly, the amount of the Pho89 protein was determined by immunoblot analysis of *snf1*, *rim101*, and *pho4* mutants. As shown in Fig. 4C, the different mutations decreased the amount of the transporter, although the pattern was not identical. For instance, mutation of *RIM101* barely affected the early peak of Pho89 accumulation, although it resulted in a faster-than-normal decrease at longer times. In parallel experiments, we also observed that deletion of *SNF1* or *REG1* did not alter the expression of the Pho84 transporter in response to low P_i, confirming the absence of Snf1-mediated regulation in response to P_i starvation (51, 52). No major changes in the accumulation pattern of Pho84 in the *snf1* mutant were observed, although a slight decrease in the overall levels of the transporter was detected in *reg1* cells subjected to alkaline-pH stress (not shown).

Mig2 is phosphorylated in response to high-pH stress and contributes to *PHO89* regulation. We next concentrated on the role of Snf1 in *PHO89* regulation. To corroborate the involvement of the kinase in the control of this promoter, we tested the *PHO89* reporter in a *reg1* strain, lacking the regulatory subunit of the Glc7 phosphatase, in which Snf1 cannot be dephosphorylated and, therefore, is hyperactive. The results (Fig. 5A) showed a substantial constitutive induction of the reporter in the *reg1* strain, which was further increased (4-fold) upon high-pH stress. The exacerbated response of the *reg1* strain was completely abolished by deletion of *SNF1*, suggesting that it was caused by hyperactivation of the kinase.

Snf1 can regulate Mig1 activity by phosphorylating the repressor and promoting exit from the nucleus under conditions of glucose scarcity (see reference 53 for a review). To test the possible contribution of Mig1 and its structurally and functionally related repressor Mig2, we measured the response of the pPHO89-LacZ reporter to high pH in cells lacking these repressors in the presence or absence of Snf1 (Fig. 5B). The absence of Mig2 was already detectable in nonstressed cells (1.41 ± 0.17 units for the wild type and 1.73 ± 0.18, 4.51 ± 0.23, and 4.90 ± 0.26 units for the *mig1*,

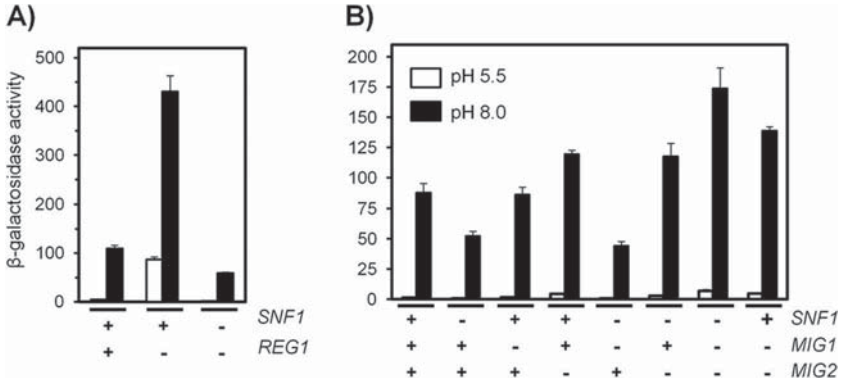

FIG 5 Regulation of *PHO89* expression by Snf1 may be mediated by Mig2. (A) Wild-type strain DBY746 and its *reg1* and *reg1 snf1* derivatives were transformed with pPHO89-LacZ and subjected to high-pH stress (pH 8.0) (black bars) prior determination of β-galactosidase activity. (B) pPHO89-LacZ activity was measured in wild-type strain DBY746 and in strains containing different combinations of the *snf1*, *mig1*, and *mig2* mutations. Data are means ± standard errors of the means from 9 to 12 experiments.

mig2, and *mig1 mig2* mutants, respectively). These results were indicative of a more prominent role for Mig2 in repression of the *PHO89* promoter. As shown in Fig. 5B, deletion of *MIG1* did not affect the response to alkaline stress, whereas a lack of Mig2 resulted in augmented promoter activity. Deletion of both repressors increased the response observed for the *mig2* mutant only slightly. The response of the *snf1 mig1* mutant was identical to that observed in *snf1* cells, whereas cells lacking both *snf1* and *mig2* showed an induction equivalent to that displayed by the *mig2* strain. These results indicate that Mig2, but not Mig1, represses the *PHO89* promoter and that high-pH-induced activation of Snf1 might relieve the repression caused by Mig2. Remarkably, we observed an unexpected higher induction in the *snf1 mig1 mig2* strain than in the *mig1 mig2* double mutant. It should also be noted that mutation of *reg1* generates a stronger derepression of the *PHO89* promoter than that achieved by deletion of both the *MIG1* and *MIG2* repressors.

It has been reported that activation of Snf1 in response to glucose starvation results in Mig1 phosphorylation (54–57). In view of the apparent absence of a role for Mig1 in the regulation of *PHO89* upon high-pH stress, we resorted to the use of a HA-tagged version of the repressor and immunoblot techniques to test whether alkalinization of the medium would result in Mig1 phosphorylation. As shown in Fig. S2A in the supplemental material, exposure to alkaline pH resulted in the rapid appearance of slower-migrating forms, indicative of hyperphosphorylated versions of the protein. This was a transient effect, since after 10 min of stress, Mig1 returned to its initial mobility. The shift was completely abolished when samples were treated prior to SDS-PAGE with alkaline phosphatase in the absence of EDTA (which blocks the phosphatase activity). The Mig1 band shift was not detected in cells lacking the Snf1 kinase, indicating a requirement for Snf1 function for alkali-induced phosphorylation of the repressor. Similarly, we investigated whether alkaline-pH stress could affect Mig1 subcellular localization by using a chromosomally integrated construct expressing a GFP-fused version of this repressor. We observed that Mig1 was mostly nuclear in nonstressed cells and that only 3 min after switching to pH 8.0, most of the fluorescence was distributed in the cytosol (see Fig. S2B in the supplemental material). This shift was transient, since after 10 min of stress, Mig1 was again concentrated in the nucleus. Therefore, alkaline-pH stress promotes transient Snf1-mediated phosphorylation and a nuclear-cytoplasmic shift of Mig1, although this does not translate into regulation of the *PHO89* promoter.

We then repeated these experiments using an HA-tagged version of Mig2. As shown in Fig. 6A, the tagged protein appeared as a doublet at ~55 kDa that very rapidly (2 min) suffered a marked shift to slower migration forms upon high-pH stress. After 10 to 15 min of stress, the pattern reversed to the original profile. Treatment of the samples with alkaline phosphatase prior to electrophoresis abolished the observed shift. When the experiment was repeated in cells lacking the Snf1 kinase, the initial mobility shift was essentially absent. For evaluation of the eventual nuclear-cytoplasmic shift of Mig2 in response to high-pH stress, we integrated a gene encoding a GFP–C-terminal fusion into the *MIG2* locus of strain BY4741. However, the fluorescent signal was too low. For unequivocal localization, we constructed this fusion in the DBY746 background, which yielded a better signal. The experiments showed that the transient phosphorylation of Mig2 correlated well with the transfer of the GFP-tagged protein from the nucleus to the cytosol and the subsequent return (10 to 15 min) to its original nuclear localization (Fig. 6B). Remarkably, localization of Mig2 was essentially cytosolic in a *reg1* mutant, even at pH 5.8 (Fig. 6B), and it was not altered by the shift to high pH (not shown). We then monitored the kinetics of phosphorylation of Snf1 in the wild-type strain exposed to pH 8.0. As shown in Fig. 6C, Snf1 is almost immediately phosphorylated in response to high-pH stress in a transient fashion, returning to baseline levels 15 to 20 min after the stress. Remarkably, in *reg1* cells, phosphorylated Snf1 was already detected in the absence of stress. Phosphorylation was further increased by high-pH stress, and the phosphorylation levels remained high during the entire experiment. Phosphorylation of Snf1 was not induced by shifting cells to low-P_i (100 μM) medium (not shown). Altogether, our results indicate that both Mig1 and Mig2 can be rapidly and transiently phosphorylated in response to alkaline stress in an Snf1-dependent manner, although only Mig2 contributes to *PHO89* regulation.

Combined regulation of *PHO89* by Snf1 and Rim101 through Nrg1 and Nrg2. The potential presence of Nrg1/Nrg2 consensus sites in the *PHO89* promoter prompted us to investigate, with the aid of the pPHO89-LacZ reporter, the possible role

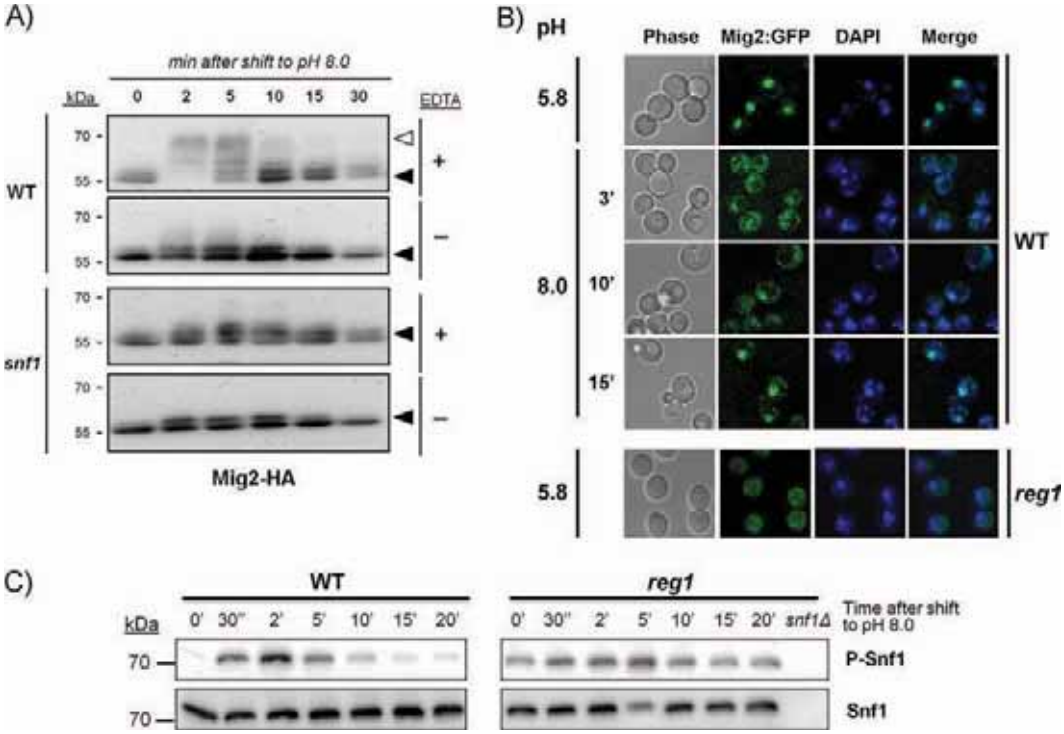

FIG 6 High-pH stress induces transient phosphorylation and nuclear-cytoplasmic shift of Mig2. (A) A chromosomally encoded copy of Mig2 including a C-terminal 3×HA epitope tag was introduced into cells of wild-type strain BY4741 and its *snf1* derivative. After cells were exposed to pH 8.0 for the indicated times, extracts were prepared and subjected to SDS-PAGE (8% polyacrylamide gels) prior to treatment with alkaline phosphatase in the absence (−) or in the presence (+) (to prevent the action of the phosphatase) of 50 mM EDTA. Immunoblot assays were performed by using anti-HA antibodies. The open triangle denotes slower (more-phosphorylated) species. (B) Strains SP048 (wild type) and ASC34 (*reg1*) containing chromosomally encoded C-terminal fusions of GFP with Mig2 were shifted to pH 8.0, and the localization of the repressor was monitored by fluorescence confocal microscopy (only pH 5.8 is shown for ASC34). Nuclei were stained with DAPI to illustrate the nuclear colocalization of the GFP and DAPI signals (merged). (C) Strains DBY746 (wild type) and RSC89 (*reg1*) were subjected to an alkaline shift for the indicated periods, and samples (10 μl) were processed for SDS-PAGE (10% gels) and immunoblotting using anti-phospho-Thr172-AMPK (phosphorylated Snf1 [P-Snf1]) or anti-His (Snf1 protein) antibodies. An extract from strain RSC10 (*snf1*Δ) is included as a negative control.

of these regulatory proteins in high-pH stress. As presented in Fig. 7A, cells lacking both Nrg1 and Nrg2 showed a detectable increase in *PHO89* promoter activity (5.5- ± 1.5-fold) under noninduced conditions, suggesting that these repressors could control *PHO89* expression. Upon high-pH stress, the absence of Nrg1 or Nrg2 resulted in a roughly similar increase in reporter activity compared with the wild-type strain, and deletion of both genes yielded a further increase in the response, indicating that both components could have a physiological role. The response of the promoter in *snf1 nrg1* and *snf1 nrg2* cells was intermediate between that observed for *snf1* cells and that observed for wild-type cells, whereas the activation level for the *snf1 nrg1 nrg2* triple mutant was identical to that of the wild-type strain and therefore was not as high as that for the *nrg1 nrg2* mutant. This could be explained by assuming that even if Snf1 can control Nrg1/2 function, in the absence of Snf1, the *PHO89* promoter still suffers Mig2-mediated repression. In agreement with this notion, in an *nrg1 nrg2 mig1 mig2* quadruple mutant strain, we observed a stronger response than in the *nrg1 nrg2* strain (Fig. 7A).

We next investigated the mechanism by which Rim101 might regulate *PHO89* induction in response to high-pH stress. Since Rim101 is known to control Nrg1 and Nrg2, we combined the *rim101* mutation with mutation of *NRG1* and/or *NRG2* and compared the induction of the pPHO89-LacZ reporter. We observed that a lack of Nrg1 or Nrg2 causes a similarly moderate increase in the response of the reporter to high pH, which is further enhanced by removal of both repressors (Fig. 7B). However, we observed that the response in a *rim101 nrg1* background was similar to that of the wild-type strain, whereas the induction level of the *rim101 nrg2* mutant was only slightly higher than that of the *rim101* strain. This suggests that the input on the *PHO89* promoter mediated by Rim101 in response to high-pH stress would involve preferentially Nrg1 over Nrg2. We also investigated the subcellular localization of Nrg1 in response to alkalinization of the medium using an Nrg1-GFP fusion (not shown) and found that the repressor does not leave the nucleus in response to the stress. This is reminiscent of the previously reported observation that the nuclear localization of Nrg1 is not regulated by the carbon source (58).

The *PHO89* promoter is high-pH but not salt responsive. Exposure of yeast cells to high concentrations of salt triggers the activation of the calcineurin and Snf1 pathways (59–61). However, we could not find evidence in the literature for induction of *PHO89* upon salt stress. To verify this point, we exposed wild-type cells carrying the pPHO89-LacZ reporter to mild (0.4 M) or strong (0.8 M) NaCl stress and compared the activities of the promoter in cells challenged by pH 8.0. As shown in Fig. S3 in the supplemental material, salt stress barely affected expression from the *PHO89* promoter. In contrast, reporters containing the entire

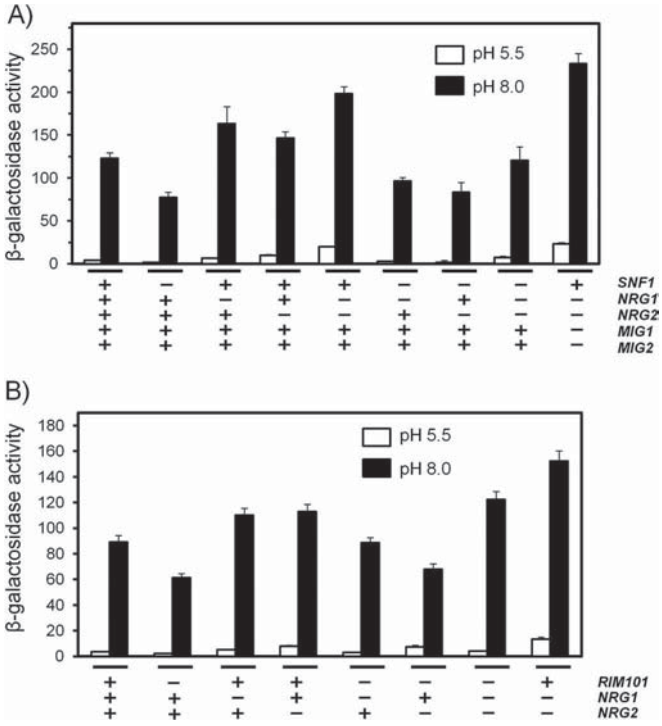

FIG 7 Snf1 and Rim101 control *PHO89* expression through Mig2 and Nrg1. The indicated strains were transformed with plasmid pPHO89-LacZ. Exponential cultures were switched to pH 8.0, and β-galactosidase activity was determined after 90 min. Data are means ± standard errors of the means from 12 to 15 experiments (A) or 20 experiments (B).

ENA1 promoter or a synthetic tandem arrangement of CDREs, which were introduced into the experiment as positive controls, were induced by both high-pH and salt stress conditions. We then examined if salt stress could affect the phosphorylation of Mig2. In Fig. S4 in the supplemental material, we show that the electrophoretic mobility of HA-tagged Mig2 was unchanged upon exposure to 0.8 M NaCl, indicating that salt stress does not trigger the phosphorylation of the repressor.

Evidence for functional coupling between Pho89 and Ena1. The regulatory network described above, involving calcineurin, Snf1, Rim101, Mig2, and Nrg1/Nrg2, was essentially identical to that reported several years ago by our laboratory regarding the regulation of the monovalent cation ATPase gene *ENA1* (24), thus suggesting the possibility of synchronic regulation. To test this, we determined the levels of mRNA of *PHO89* and *ENA1* and plotted the values against time after the shift to high pH (8.0). As shown in Fig. 8A, in both cases, the amounts of mRNA increased rapidly, with a peak ~10 to 15 min after alkaline stress, and then decreased nearly in parallel. Similarly, a peak of Pho89 protein accumulation was produced 15 to 20 min after the mRNA peak, roughly at the same time that Ena1 accumulation reached its maximum. It is worth noting that whereas Pho89 levels declined quite sharply, the decrease of Ena1 accumulation was slower, allowing detection of significant levels of the transporter even 3 h after the onset of stress. These results indicated that Ena1 and Pho89 are produced at nearly the same time upon exposure to high-pH stress. Since Ena1 actively extrudes Na^+ cations and Pho89 requires monovalent cations (preferably Na^+) for effective phosphate cotransport, we considered the possibility that this coordinated regulation is at the basis of a functional link between both activities. To test this possibility, we compared the growth rates of strains lacking the high-affinity phosphate transporters and/or the cation ATPase gene under different pH and phosphate availability conditions. As shown in Fig. 8B, at standard (acidic) pH and in the presence of limiting amounts of phosphate (0.2 mM), the *pho84*, *ena1 pho84*, and *pho84 pho89* strains could not proliferate. Under the same conditions, but at pH 7.6, the *pho84* mutant grew reasonably well, likely due to the activity of Pho89, which would be induced by the lack of phosphate and the alkaline environment and, as shown here (see Fig. S1 in the supplemental material), enhanced by the absence of Pho84. As expected, further deletion of *PHO89* blocked proliferation. Remarkably, virtually the same effect was achieved by deleting the *ENA1* to *-4* cluster, thus blocking the ability to extrude Na^+ cations at alkaline pH (it should be noted that *ENA1* represents the main extrusion system in the cluster and the only one induced by high pH). Therefore, the lack of *ENA1* mimics the effect of mutating *PHO89* in a *pho84* background, thus phenocopying the situation of full deficiency of high-affinity phosphate transporters.

Shortly after the discovery of *PHO89*, it was proposed that the activity of *ENA1* might serve as a Na^+ motive force for phosphate transport through Pho89 (6). However, our plates shown in Fig. 8B contained 5 mM Na^+, an external concentration sufficient to drive substantial phosphate influx through Pho89 (5, 8). Nevertheless, we designed an experiment to test this possibility. To this end, *pho84*, *ena1*, *ena1 pho84*, and *ena1 pho89* cells were grown at pH 7.2 in the presence of 5 mM or 0.2 mM NaCl with either 5 mM or 0.2 mM P_i in the medium, and the 5/0.2 mM Na^+ growth ratio was calculated and plotted. As shown in Fig. S5 in the supplemental material, *ena1 pho84* cells grew worse at 5 mM than at 0.2 mM NaCl, and this effect was independent of the amount of P_i present in the medium, while the other mutants grew equally well under both NaCl conditions. This result raised the possibility that the growth defect of the *ena1 pho84* mutant at alkaline pH is derived not from the lack of enough Na^+ to sustain Pho89-mediated P_i uptake but from the intracellular accumulation of toxic Na^+ cations accompanying P_i influx, which could not be eliminated in the absence of Ena1. To assess this possibility, the relevant strains were grown for 60 min in low-P_i medium (0.2 mM) in the presence of 5 mM NaCl and then shifted to pH 7.8, and samples were taken periodically and processed for determination of intracellular Na^+ content. As shown in Fig. 8C, wild-type or *pho84* cells did not significantly accumulate Na^+ even after 8 h of growth. Mutation of *ENA1* provoked a moderate, time-dependent intracellular accumulation of the cation that was distinctly augmented upon deletion of *PHO84*. This effect was interpreted as the result of increased Pho89 activity due to the absence of Pho84-mediated transport. This hypothesis was reinforced by the observation that an *ena1 pho89* mutant accumulated very little intracellular Na^+, clearly below the levels measured for the *ena1* strain. Collectively, our results indicate that the accumulation of intracellular Na^+ as a result of Pho89 activity can be detrimental in the absence of Ena1 and suggest that, under these conditions, the activity of the ATPase may be instrumental as a detoxification mechanism (Fig. 8D).

DISCUSSION

The capacity of yeast cells to survive and grow under changing environmental conditions is of widespread importance in the fun-

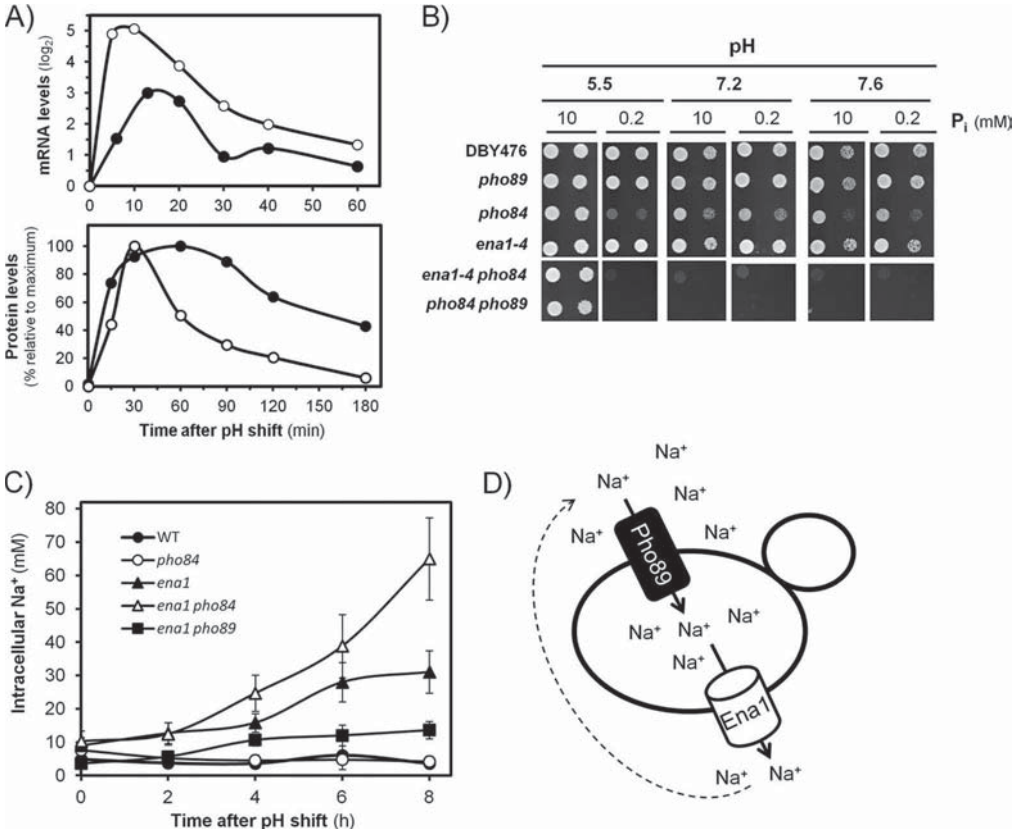

FIG 8 Coregulation of Pho89 and Ena1 expression allows functional coupling between both proteins under conditions of high-pH stress. (A) Concerted coregulation of Pho89 (open circles) and Ena1 (closed circles) expression. Shown are time courses for *ENA1* and *PHO89* mRNA accumulation (top) or protein accumulation expressed as a percentage over the maximum signal (bottom) after shifting cells from pH 5.5 to 8.0 (see Materials and Methods for details). (B) The indicated strains at an OD_{660} of 0.05, plus a 10-fold dilution, were spotted onto YNB-based medium (lacking phosphate and sodium) agar plates supplemented with 5 mM NaCl and the indicated amounts of potassium phosphate and adjusted to different pHs. Growth was monitored after 3 days. (C) The indicated strains were shifted for 1 h to low-P_i (0.2 mM) medium at pH 5.5 and then shifted to pH 7.8, always in the presence of 5 mM NaCl. Samples were taken at the indicated periods and processed for determination of the intracellular Na^+ content. Data are means ± standard errors of the means from 4 to 8 independent experiments. (D) Illustration of the proposed functional link between Ena1 and Pho89 under high-pH stress (see the text for explanations).

gal pathogenicity of animals and plants and also from a biotechnological view (fungal production of antibiotics, secreted enzymes, and toxic compounds). Budding yeast has been shown to be an excellent model to study cellular strategies used to maintain appropriate intracellular ion concentrations and intracellular pH, among other important cellular parameters, when exposed to detrimental external conditions. However, little is known about the functional interactions between different ion transporters and how these possible interactions are sustained by common signaling pathways. Our work shows that Ena1 and Pho89 transporters are subjected to the same regulatory network, thus allowing coordinated expression and functional coupling upon high-pH stress.

PHO84 and *PHO89* encode proteins that constitute the high-affinity phosphate transport system in budding yeast, and they are considered members of the *PHO* regulon, under the control of the Pho4/Pho2 transcriptional activators. We show here that under phosphate starvation, Pho84 accumulates much faster than Pho89, whereas under high-pH stress, a condition previously known to induce *PHO84* and *PHO89* expression (15, 16, 22), induction of *PHO89* occurs earlier. Differential expression kinetics for *PHO84* and *PHO89* were also observed in yeast cells exposed to the cell wall-damaging agent Congo red, with the *PHO89* response being faster than that of *PHO84* (62), and it is known that mutation of the Ptc1 phosphatase gene or Mg^{2+} deprivation results in the induction of *PHO89* but not of *PHO84* (63, 64). In contrast, we recently observed that deprivation of potassium induces *PHO84* but not *PHO89* expression (65). Therefore, it seems evident that in spite of being cataloged as members of the *PHO* regulon, *PHO84* and *PHO89* exhibit distinct regulatory properties. The network regulating *PHO89* expression described in our work allows explanation of these differences.

We demonstrate that in cells exposed to alkaline-pH stress, *PHO89* is under the control of a complex signaling network involving (in addition to Pho4) calcineurin, Snf1, and Rim101 (see Fig. 9 for a schematic depiction of the proposed network). Previous evidence suggested that high-pH induction of *PHO89*, but not that of *PHO84*, was influenced by the presence of calcineurin. We confirm here that the Ca^{2+}-activated phosphatase calcineurin is a major regulator of *PHO89* upon alkaline-pH stress. This is explained by the previously reported observation that exposure to high pH triggers a very fast entry of calcium cations into the cell from the extracellular medium (16). Activation of calcineurin by high pH results in dephosphorylation of Crz1 and very fast entry (2 to 5 min) of the transcription factor into the nucleus (49; our

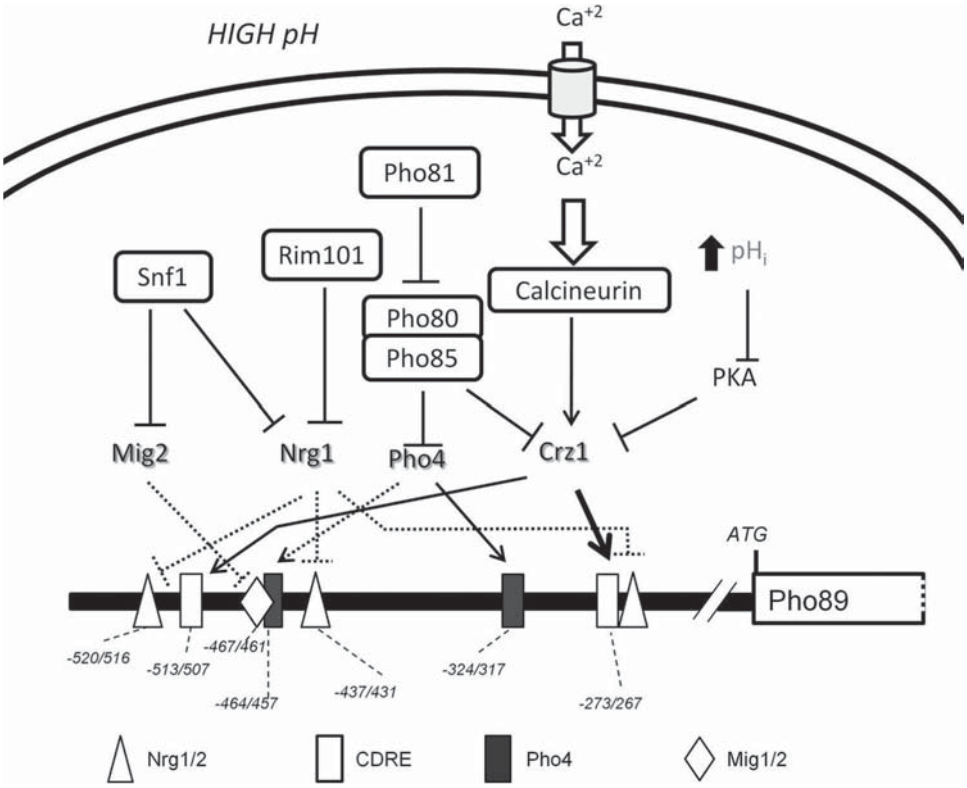

FIG 9 Schematic depiction of regulatory inputs acting on the *PHO89* promoter upon high-pH stress. Elements upstream of Snf1, Rim101, Pho81, or calcineurin are not included for clarity. Discontinuous lines indicate possible physical interactions not experimentally tested. The functional Pho4 consensus site is deduced from recently reported genome-wide data (85).

unpublished results). In agreement with this kinetic, we observed recruitment of Crz1 to the *PHO89* promoter in the first few minutes after shifting cells to alkaline pH. We also identify, by mutational analysis, a CDRE responsible for most of the regulatory properties of calcineurin. The evidence that *PHO89* expression is potently controlled by calcineurin allows an explanation for why the basal expression level of *PHO89* is higher than normal (and sensitive to the calcineurin inhibitor FK506) in cells deficient in the protein phosphatase Ppz1, since these mutants have a constitutively activated calcineurin pathway (66). Moreover, the calcineurin-mediated activation of Crz1 would be reinforced by both the inhibition of the protein kinase A (PKA) pathway (21) and the activation of the *PHO* pathway under high-pH stress, since protein kinase A and Pho85 are known to phosphorylate Crz1 (67, 68), thus contributing to its inactivation (Fig. 9). Interestingly, the regulation of *PHO89* by calcium/calcineurin may be a common trait in fungi, since a recent report showed an important role of calcium influx in the alkaline stress response, which was also Crz1 dependent, in *Candida albicans* (69). This control mechanism is reminiscent of the induced expression of the sodium-dependent phosphate cotransporter Pit-1, the human homolog of Pho89, by long-term calcium treatment in smooth muscle cells (70).

Exposure of yeast cells to high-salt stress also increases intracellular calcium concentrations (71, 72) and activates calcineurin. Activation of calcineurin is responsible for part of the transcriptional response of salt-induced genes, such as the Na^+-ATPase *ENA1*. However, our results indicate that saline stress does not induce *PHO89* expression. This suggests that either the potency of the calcium signal generated by salt stress is insufficient to activate *PHO89* or efficient induction of this gene also requires the concomitant derepression of its promoter, not triggered by salt stress. In this regard, it is interesting that we did not observe phosphorylation of Mig2 upon exposure to high salt (see Fig. S4 in the supplemental material), suggesting that this repressor may remain bound to the *PHO89* promoter under this kind of stress.

It has been reported that Snf1, which is cytosolic in unstressed cells, becomes enriched in the nucleus when cells are subjected to alkaline pH (59). Under glucose-limiting conditions, Snf1 phosphorylates Mig1 and promotes its export from the nucleus, thus relieving transcriptional repression of a set of glucose-repressed genes. We observed that Mig1 can be rapidly and transiently phosphorylated in response to alkaline stress in an Snf1-dependent manner and that Mig1 is rapidly released from the nucleus upon shifting cells to high pH. This is in contrast to what was reported previously for high-salt stress, in which Snf1 activation is not followed by Mig1 phosphorylation (73, 74). However, our data indicate that Mig1 is barely relevant in repressing *PHO89* under normal growth conditions and plays a small role in the expression change of the transporter gene upon high-pH induction. Therefore, it can be assumed that either Mig1 is not bound to the *PHO89* promoter under standard growth conditions or it is unable to repress *PHO89* expression. Here we present evidence that Mig2, a homolog of Mig1, is also rapidly phosphorylated in an Snf1-dependent way in response to high-pH stress. This is remarkable because for many years, it has been generally accepted that, in contrast to Mig1, Mig2 is not a substrate for the Snf1 kinase (75,

76). Recent work proposed that galactose induces fast degradation of Mig2 by a mechanism that involves Snf1-dependent Mig2 phosphorylation (77), although it is worth noting that those authors observed an almost complete depletion of Mig2 after only 20 min upon shifting of cells to galactose, whereas in our hands, the amount of Mig2 did not significantly change after 30 min of alkaline stress. In any case, our data support a role for Mig2 in repression of *PHO89* expression, and it is reasonable to assume that activation of Snf1 results in a relief of such repression. In this regard, previously reported transcriptomic data based on DNA microarray studies pointed to a strong derepression of *PHO89* in a *mig1 mig2* mutant (78), whereas the expression of *PHO89* in the *mig1* mutant was similar to that of the wild type. Although those authors postulated a redundant role for *mig1* and *mig2* in the regulation of *PHO89*, their results also seem consistent with a preeminent role for Mig2 in the control of *PHO89* expression. In conclusion, we postulate that Mig2, but not Mig1, seems to be relevant for mediating Snf1-controlled induction of *PHO89* upon high-pH stress.

It has been shown that Snf1 interacts with the repressor Nrg1 and its closely related homolog Nrg2 (58), and both repressors have been reported to mediate Snf1-dependent responses (79, 80). *NRG1* expression is downregulated by high-pH stress, and deletion of this gene results in increased *ENA1* expression levels under these conditions (23, 24). In addition, Nrg1 is also under the control of Rim101 in response to salt and alkaline-pH stresses (22–24). We show here that Nrg1 is likely repressing *PHO89* expression and that high-pH stress leads to derepression of the promoter in a way that involves combined control by both Snf1 and Rim101. It must be noted that in contrast to other organisms such as *Aspergillus*, where Rim101 directly controls the expression of alkali-inducible genes (see reference 81 for a review), in budding yeast, the effect seems to be indirect: the activation of Rim101 represses the expression of Nrg1, thus relieving the repression of Nrg1 target genes such as *ENA1* (23) or *PHO89* (this work). This indirect control, which would involve a 2-step mechanism, would explain why deletion of *RIM101* affects the late response of *PHO89* but not the early peak of accumulation of Pho89 mRNA or protein (Fig. 4B and C).

The involvement of Snf1 in the regulation of *PHO89* is further reinforced by the observation that deletion of *REG1* resulted in a dramatic increase of *PHO89* promoter activity under both basal and high-pH stress conditions that is fully Snf1 dependent. Mutation of *REG1* causes hyperactivation of Snf1 (41), and we observed constitutive and sustained phosphorylation of Snf1 and a cytosolic localization of Mig2 in the *reg1* mutant (Fig. 6B and C). Remarkably, the potent derepression of *PHO89* in *reg1* cells was substantially greater than that achieved in the *mig1 mig2 nrg1 nrg2* quadruple mutant. Therefore, the phenomenon cannot be explained only by Snf1-mediated inhibition of the repressor's function. Although we do not have a definite explanation, this observation is not totally surprising, since existing evidence indicates that Snf1 affects multiple steps in gene regulation, including transcription factor binding, RNA polymerase II activity, and cytoplasmic mRNA stability (82).

Alkaline-pH stress results in the transcriptional induction of the Ena1 Na^+-ATPase, which actively extrudes Na^+ cations (15, 26, 35, 61), whereas Pho89 is a Na^+-driven phosphate transporter active at alkaline pH (5). Previous results from our laboratory (24) demonstrated that alkaline stress induction of *ENA1* is mediated by three different pathways, involving the activation of calcineurin, Rim101 (through Nrg1), and Snf1 (through Nrg1 and Mig2). Here we provide experimental evidence that *ENA1* and *PHO89* are under the control of the same regulatory network (Fig. 9), thus allowing the coordinate expression of both proteins in response to high-pH stress (Fig. 8A). In addition, we demonstrate that under alkaline-pH conditions, mutation of *ENA1* in a *pho84* background results in the inability to grow in phosphate-depleted medium. This strongly suggests that the functions of Ena1 and Pho89 are linked. It should be noted that simply deleting *ENA1* and growing cells in low-phosphate medium does not allow testing of this hypothesis, since Pho84 has a higher transport capacity than Pho89, and although it is optimally active at acidic pH, it retains a significant activity at neutral or even mild alkaline pH (8).

Shortly after the discovery of *PHO89*, it was proposed that Ena1 could provide a motive force for Pho89-mediated phosphate transport (6). While this notion might still hold in some circumstances, alternative scenarios can be devised. Our observation that increasing the amount of Na^+ in the medium (which should diminish the role of Ena1 as a provider of driving force) did not improve growth when *ENA1* was deleted in the *pho84* background suggests that the main role of Ena1 would be to avoid the accumulation of Na^+ cations entering through Pho89, instead of supplying Na^+ cations for Pho89-mediated P_i entry. This scenario is supported by the observation that the *ena1 pho84* strain accumulated high levels of Na^+ but that the *ena1 pho89* strain did not, pointing to the fact that the observed accumulation of Na^+ is the result of the Pho89 activity. This increase in Na^+ content could be detrimental to the cell for at least two reasons: (i) because of the recognized toxicity of Na^+ cations and (ii) because the increase in intracellular Na^+ levels would abolish or even reverse the extracellular/intracellular cation gradient that is necessary to support effective P_i entry through Pho89 (Fig. 8D). In any case, the existence of an identical high-pH-responsive signaling network controlling both genes would serve to ensure the well-timed expression of these transporters, which would be clearly advantageous for the cell to thrive in such adverse environments. Pho89-like transporters are widely spread among eukaryotes, whereas Ena1-like ATPases are found in all fungi and are extensively present in bryophyte and parasitic protozoa (83). The requirement for Ena1 to grow at alkaline pH (even in the absence of salt stress) has been proven for very diverse fungi, such as *Ustilago maydis* and *Cryptococcus neoformans* (see reference 83 and references therein), and the induction of *ENA1* genes in response to a shift to high pH not only is a fungal characteristic but also is observed in bryophytes (84). Therefore, the functional and regulatory coupling between Pho89 and Ena1 transporters reported here for budding yeast might be conserved through evolution.

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We declare that we do not have any conflict of interest.

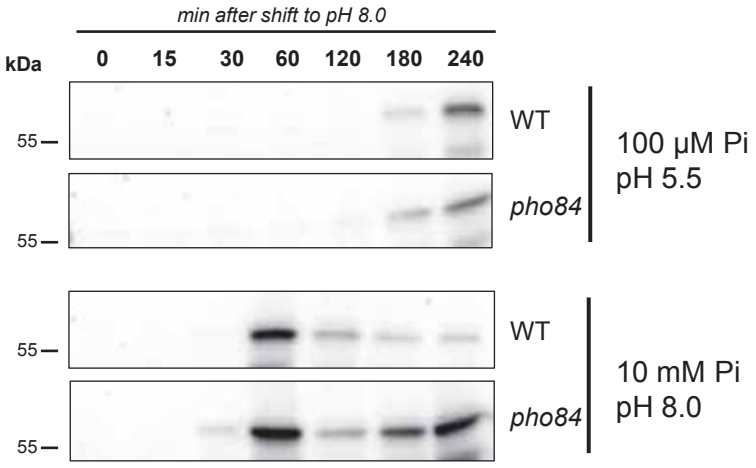
REFERENCES

1. **Bun-ya M, Shikata K, Nakade S, Yompakdee C, Harashima S, Oshima Y.** 1996. Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. Curr. Genet. **29:**344–351. http://dx.doi.org/10.1007/s002940050055.
2. **Ghillebert R, Swinnen E, De Snijder P, Smets B, Winderickx J.** 2011. Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in Saccharomyces cerevisiae. Biochem. J. **434:**243–251. http://dx.doi.org/10.1042/BJ20101118.
3. **Wykoff DD, O'Shea EK.** 2001. Phosphate transport and sensing in Saccharomyces cerevisiae. Genetics **159:**1491–1499.
4. **Bun-ya M, Nishimura M, Harashima S, Oshima Y.** 1991. The PHO84 gene of Saccharomyces cerevisiae encodes an inorganic phosphate transporter. Mol. Cell. Biol. **11:**3229–3238.
5. **Martinez P, Persson BL.** 1998. Identification, cloning and characterization of a derepressible Na+-coupled phosphate transporter in Saccharomyces cerevisiae. Mol. Gen. Genet. **258:**628–638. http://dx.doi.org/10.1007/s004380050776.
6. **Persson BL, Petersson J, Fristedt U, Weinander R, Berhe A, Pattison J.** 1999. Phosphate permeases of Saccharomyces cerevisiae: structure, function and regulation. Biochim. Biophys. Acta **1422:**255–272. http://dx.doi.org/10.1016/S0304-4157(99)00010-6.
7. **Pattison-Granberg J, Persson BL.** 2000. Regulation of cation-coupled high-affinity phosphate uptake in the yeast Saccharomyces cerevisiae. J. Bacteriol. **182:**5017–5019. http://dx.doi.org/10.1128/JB.182.17.5017-5019.2000.
8. **Zvyagilskaya RA, Lundh F, Samyn D, Pattison-Granberg J, Mouillon JM, Popova Y, Thevelein JM, Persson BL.** 2008. Characterization of the Pho89 phosphate transporter by functional hyperexpression in Saccharomyces cerevisiae. FEMS Yeast Res. **8:**685–696. http://dx.doi.org/10.1111/j.1567-1364.2008.00408.x.
9. **Ogawa N, Saitoh H, Miura K, Magbanua JP, Bun-ya M, Harashima S, Oshima Y.** 1995. Structure and distribution of specific cis-elements for transcriptional regulation of PHO84 in Saccharomyces cerevisiae. Mol. Gen. Genet. **249:**406–416.
10. **Persson BL, Lagerstedt JO, Pratt JR, Pattison-Granberg J, Lundh K, Shokrollahzadeh S, Lundh F.** 2003. Regulation of phosphate acquisition in Saccharomyces cerevisiae. Curr. Genet. **43:**225–244. http://dx.doi.org/10.1007/s00294-003-0400-9.
11. **Auesukaree C, Homma T, Kaneko Y, Harashima S.** 2003. Transcriptional regulation of phosphate-responsive genes in low-affinity phosphate-transporter-defective mutants in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. **306:**843–850. http://dx.doi.org/10.1016/S0006-291X(03)01068-4.
12. **Ogawa N, DeRisi J, Brown PO.** 2000. New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol. Biol. Cell **11:**4309–4321. http://dx.doi.org/10.1091/mbc.11.12.4309.
13. **Kaffman A, Herskowitz I, Tjian R, O'Shea EK.** 1994. Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. Science **263:**1153–1156. http://dx.doi.org/10.1126/science.8108735.
14. **Toh-e A, Tanaka K, Uesono Y, Wickner RB.** 1988. PHO85, a negative regulator of the PHO system, is a homolog of the protein kinase gene, CDC28, of Saccharomyces cerevisiae. Mol. Gen. Genet. **214:**162–164. http://dx.doi.org/10.1007/BF00340196.
15. **Serrano R, Ruiz A, Bernal D, Chambers JR, Arino J.** 2002. The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. Mol. Microbiol. **46:**1319–1333. http://dx.doi.org/10.1046/j.1365-2958.2002.03246.x.
16. **Viladevall L, Serrano R, Ruiz A, Domenech G, Giraldo J, Barcelo A, Arino J.** 2004. Characterization of the calcium-mediated response to alkaline stress in *Saccharomyces cerevisiae*. J. Biol. Chem. **279:**43614–43624. http://dx.doi.org/10.1074/jbc.M403606200.
17. **Cyert MS.** 2003. Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. Biochem. Biophys. Res. Commun. **311:**1143–1150. http://dx.doi.org/10.1016/S0006-291X(03)01552-3.
18. **Werner A, Kinne RK.** 2001. Evolution of the Na-P(i) cotransport systems. Am. J. Physiol. Regul. Integr. Comp. Physiol. **280:**R301–R312.
19. **Serrano R, Martin H, Casamayor A, Arino J.** 2006. Signaling alkaline pH stress in the yeast Saccharomyces cerevisiae through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. J. Biol. Chem. **281:**39785–39795. http://dx.doi.org/10.1074/jbc.M604497200.
20. **Casamayor A, Serrano R, Platara M, Casado C, Ruiz A, Arino J.** 2012. The role of the Snf1 kinase in the adaptive response of Saccharomyces cerevisiae to alkaline pH stress. Biochem. J. **444:**39–49. http://dx.doi.org/10.1042/BJ20112099.
21. **Casado C, Gonzalez A, Platara M, Ruiz A, Arino J.** 2011. The role of the protein kinase A pathway in the response to alkaline pH stress in yeast. Biochem. J. **438:**523–533. http://dx.doi.org/10.1042/BJ20110607.
22. **Lamb TM, Xu W, Diamond A, Mitchell AP.** 2001. Alkaline response genes of Saccharomyces cerevisiae and their relationship to the RIM101 pathway. J. Biol. Chem. **276:**1850–1856. http://dx.doi.org/10.1074/jbc.M008381200.
23. **Lamb TM, Mitchell AP.** 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes NRG1 and SMP1 in Saccharomyces cerevisiae. Mol. Cell. Biol. **23:**677–686. http://dx.doi.org/10.1128/MCB.23.2.677-686.2003.
24. **Platara M, Ruiz A, Serrano R, Palomino A, Moreno F, Arino J.** 2006. The transcriptional response of the yeast Na+-ATPase ENA1 gene to alkaline stress involves three main signaling pathways. J. Biol. Chem. **281:**36632–36642. http://dx.doi.org/10.1074/jbc.M606483200.
25. **Ruiz A, Arino J.** 2007. Function and regulation of the Saccharomyces cerevisiae ENA sodium ATPase system. Eukaryot. Cell **6:**2175–2183. http://dx.doi.org/10.1128/EC.00337-07.
26. **Garciadeblas B, Rubio F, Quintero FJ, Banuelos MA, Haro R, Rodriguez-Navarro A.** 1993. Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. Mol. Gen. Genet. **236:**363–368.
27. **Wieland J, Nitsche AM, Strayle J, Steiner H, Rudolph HK.** 1995. The PMR2 gene cluster encodes functionally distinct isoforms of a putative Na+ pump in the yeast plasma membrane. EMBO J. **14:**3870–3882.
28. **Posas F, Camps M, Arino J.** 1995. The PPZ protein phosphatases are important determinants of salt tolerance in yeast cells. J. Biol. Chem. **270:**13036–13041. http://dx.doi.org/10.1074/jbc.270.22.13036.
29. **Haro R, Garciadeblas B, Rodriguez-Navarro A.** 1991. A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. **291:**189–191. http://dx.doi.org/10.1016/0014-5793(91)81280-L.
30. **Adams A, Gottschling DE, Kaiser CA, Stearns T.** 1997. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Davis RW.** 1999. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science **285:**901–906. http://dx.doi.org/10.1126/science.285.5429.901.
32. **Longtine MS, McKenzie A, III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953–961.
33. **Rodriguez C, Sanz P, Gancedo C.** 2003. New mutations of Saccharomyces cerevisiae that partially relieve both glucose and galactose repression activate the protein kinase Snf1. FEMS Yeast Res. **3:**77–84. http://dx.doi.org/10.1111/j.1567-1364.2003.tb00141.x.
34. **Ruiz A, González A, García-Salcedo R, Ramos J, Arino J.** 2006. Role of protein phosphatases 2C on tolerance to lithium toxicity in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **62:**263–277. http://dx.doi.org/10.1111/j.1365-2958.2006.05370.x.
35. **Alepuz PM, Cunningham KW, Estruch F.** 1997. Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated ENA1 gene. Mol. Microbiol. **26:**91–98. http://dx.doi.org/10.1046/j.1365-2958.1997.5531917.x.
36. **Cunningham KW, Fink GR.** 1996. Calcineurin inhibits VCX1-dependent H+/Ca2+ exchange and induces Ca2+ ATPases in Saccharomyces cerevisiae. Mol. Cell. Biol. **16:**2226–2237.
37. **Stathopoulos AM, Cyert MS.** 1997. Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:**3432–3444. http://dx.doi.org/10.1101/gad.11.24.3432.
38. **Horak J, Wolf DH.** 2001. Glucose-induced monoubiquitination of the

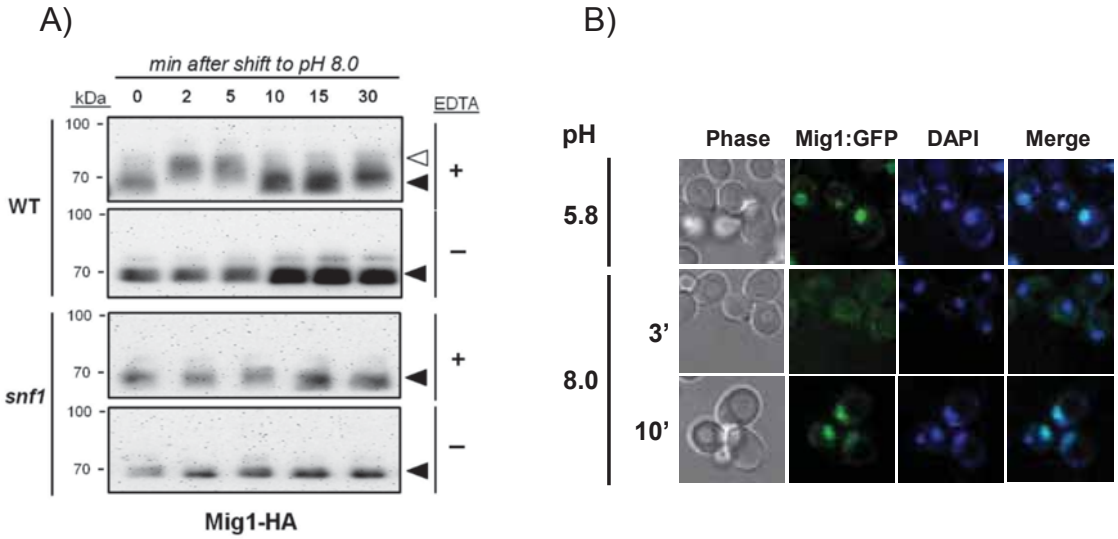
Saccharomyces cerevisiae galactose transporter is sufficient to signal its internalization. J. Bacteriol. **183:**3083–3088. http://dx.doi.org/10.1128/JB.183.10.3083-3088.2001.

39. **Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN, Loewith R.** 2007. Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol. Cell **26:**663–674. http://dx.doi.org/10.1016/j.molcel.2007.04.020.
40. **González A, Casado C, Ariño J, Casamayor A.** 2013. Ptc6 is required for proper rapamycin-induced down-regulation of the genes coding for ribosomal and rRNA processing proteins in *S. cerevisiae*. PLoS One **8:**e64470. http://dx.doi.org/10.1371/journal.pone.0064470.
41. **Orlova M, Barrett L, Kuchin S.** 2008. Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. Yeast **25:**745–754. http://dx.doi.org/10.1002/yea.1628.
42. **Langmead B, Salzberg SL.** 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods **9:**357–359. http://dx.doi.org/10.1038/nmeth.1923.
43. **Navarrete C, Petrezselyova S, Barreto L, Martinez JL, Zahradka J, Arino J, Sychrova H, Ramos J.** 2010. Lack of main K+ uptake systems in Saccharomyces cerevisiae cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions. FEMS Yeast Res. **10:**508–517. http://dx.doi.org/10.1111/j.1567-1364.2010.00630.x.
44. **Verduyn C, Postma E, Scheffers WA, Van Dijken JP.** 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast **8:**501–517. http://dx.doi.org/10.1002/yea.320080703.
45. **Thomas-Chollier M, Defrance M, Medina-Rivera A, Sand O, Herrmann C, Thieffry D, van Helden J.** 2011. RSAT 2011: regulatory sequence analysis tools. Nucleic Acids Res. **39:**W86–W91. http://dx.doi.org/10.1093/nar/gkr377.
46. **Portales-Casamar E, Thongjuea S, Kwon AT, Arenillas D, Zhao X, Valen E, Yusuf D, Lenhard B, Wasserman WW, Sandelin A.** 2010. JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. Nucleic Acids Res. **38:**D105–D110. http://dx.doi.org/10.1093/nar/gkp950.
47. **Yoshimoto H, Saltsman K, Gasch AP, Li HX, Ogawa N, Botstein D, Brown PO, Cyert MS.** 2002. Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **277:**31079–31088. http://dx.doi.org/10.1074/jbc.M202718200.
48. **Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA.** 2001. Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell **12:**323–337. http://dx.doi.org/10.1091/mbc.12.2.323.
49. **Ruiz A, Serrano R, Arino J.** 2008. Direct regulation of genes involved in glucose utilization by the calcium/calcineurin pathway. J. Biol. Chem. **283:**13923–13933. http://dx.doi.org/10.1074/jbc.M708683200.
50. **Hong SP, Leiper FC, Woods A, Carling D, Carlson M.** 2003. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc. Natl. Acad. Sci. U. S. A. **100:**8839–8843. http://dx.doi.org/10.1073/pnas.1533136100.
51. **Klosinska MM, Crutchfield CA, Bradley PH, Rabinowitz JD, Broach JR.** 2011. Yeast cells can access distinct quiescent states. Genes Dev. **25:**336–349. http://dx.doi.org/10.1101/gad.2011311.
52. **Huang D, Farkas I, Roach PJ.** 1996. Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in Saccharomyces cerevisiae. Mol. Cell. Biol. **16:**4357–4365.
53. **Santangelo GM.** 2006. Glucose signaling in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. **70:**253–282. http://dx.doi.org/10.1128/MMBR.70.1.253-282.2006.
54. **Schmidt MC, McCartney RR, Zhang X, Tillman TS, Solimeo H, Wolfl S, Almonte C, Watkins SC.** 1999. Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in Saccharomyces cerevisiae. Mol. Cell. Biol. **19:**4561–4571.
55. **Treitel MA, Kuchin S, Carlson M.** 1998. Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. Mol. Cell. Biol. **18:**6273–6280.
56. **Sutherland CM, Hawley SA, McCartney RR, Leech A, Stark MJ, Schmidt MC, Hardie DG.** 2003. Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. Curr. Biol. **13:**1299–1305. http://dx.doi.org/10.1016/S0960-9822(03)00459-7.
57. **Ostling J, Ronne H.** 1998. Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. **252:**162–168. http://dx.doi.org/10.1046/j.1432-1327.1998.2520162.x.
58. **Vyas VK, Kuchin S, Carlson M.** 2001. Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. Genetics **158:**563–572.
59. **Hong SP, Carlson M.** 2007. Regulation of snf1 protein kinase in response to environmental stress. J. Biol. Chem. **282:**16838–16845. http://dx.doi.org/10.1074/jbc.M700146200.
60. **Nakamura T, Liu Y, Hirata D, Namba H, Harada S, Hirokawa T, Miyakawa T.** 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. **12:**4063–4071.
61. **Mendoza I, Rubio F, Rodriguez-Navarro A, Pardo JM.** 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**8792–8796.
62. **Garcia R, Bermejo C, Grau C, Perez R, Rodriguez-Pena JM, Francois J, Nombela C, Arroyo J.** 2004. The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. J. Biol. Chem. **279:**15183–15195. http://dx.doi.org/10.1074/jbc.M312954200.
63. **Gonzalez A, Ruiz A, Serrano R, Arino J, Casamayor A.** 2006. Transcriptional profiling of the protein phosphatase 2C family in yeast provides insights into the unique functional roles of Ptc1. J. Biol. Chem. **281:**35057–35069. http://dx.doi.org/10.1074/jbc.M607919200.
64. **Wiesenberger G, Steinleitner K, Malli R, Graier WF, Vormann J, Schweyen RJ, Stadler JA.** 2007. Mg2+ deprivation elicits rapid Ca2+ uptake and activates Ca2+/calcineurin signaling in Saccharomyces cerevisiae. Eukaryot. Cell **6:**592–599. http://dx.doi.org/10.1128/EC.00382-06.
65. **Barreto L, Canadell D, Valverde-Saubi D, Casamayor A, Arino J.** 2012. The short-term response of yeast to potassium starvation. Environ. Microbiol. **14:**3026–3042. http://dx.doi.org/10.1111/j.1462-2920.2012.02887.x.
66. **Ruiz A, Yenush L, Arino J.** 2003. Regulation of ENA1 Na(+)-ATPase gene expression by the Ppz1 protein phosphatase is mediated by the calcineurin pathway. Eukaryot. Cell **2:**937–948. http://dx.doi.org/10.1128/EC.2.5.937-948.2003.
67. **Kafadar KA, Cyert MS.** 2004. Integration of stress responses: modulation of calcineurin signaling in Saccharomyces cerevisiae by protein kinase A. Eukaryot. Cell **3:**1147–1153. http://dx.doi.org/10.1128/EC.3.5.1147-1153.2004.
68. **Sopko R, Huang D, Preston N, Chua G, Papp B, Kafadar K, Snyder M, Oliver SG, Cyert M, Hughes TR, Boone C, Andrews B.** 2006. Mapping pathways and phenotypes by systematic gene overexpression. Mol. Cell **21:**319–330. http://dx.doi.org/10.1016/j.molcel.2005.12.011.
69. **Wang H, Liang Y, Zhang B, Zheng W, Xing L, Li M.** 2011. Alkaline stress triggers an immediate calcium fluctuation in Candida albicans mediated by Rim101p and Crz1p transcription factors. FEMS Yeast Res. **11:**430–439. http://dx.doi.org/10.1111/j.1567-1364.2011.00730.x.
70. **Yang H, Curinga G, Giachelli CM.** 2004. Elevated extracellular calcium levels induce smooth muscle cell matrix mineralization in vitro. Kidney Int. **66:**2293–2299. http://dx.doi.org/10.1111/j.1523-1755.2004.66015.x.
71. **Denis V, Cyert MS.** 2002. Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. J. Cell Biol. **156:**29–34. http://dx.doi.org/10.1083/jcb.200111004.
72. **Matsumoto TK, Ellsmore AJ, Cessna SG, Low PS, Pardo JM, Bressan RA, Hasegawa PM.** 2002. An osmotically induced cytosolic Ca2+ transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of Saccharomyces cerevisiae. J. Biol. Chem. **277:**33075–33080. http://dx.doi.org/10.1074/jbc.M205037200.
73. **McCartney RR, Schmidt MC.** 2001. Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J. Biol. Chem. **276:**36460–36466. http://dx.doi.org/10.1074/jbc.M104418200.
74. **Ye T, Elbing K, Hohmann S.** 2008. The pathway by which the yeast protein kinase Snf1p controls acquisition of sodium tolerance is different from that mediating glucose regulation. Microbiology **154:**2814–2826. http://dx.doi.org/10.1099/mic.0.2008/020149-0.
75. **Lutfiyya LL, Iyer VR, DeRisi J, DeVit MJ, Brown PO, Johnston M.** 1998. Characterization of three related glucose repressors and genes they regulate in Saccharomyces cerevisiae. Genetics **150:**1377–1391.

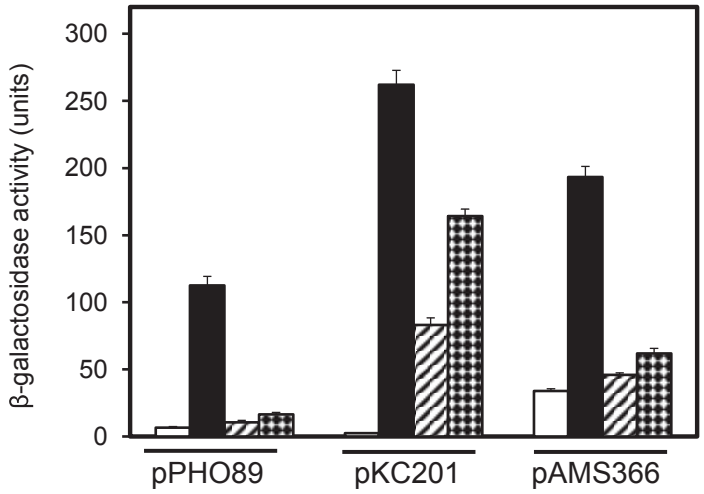
76. **Kaniak A, Xue Z, Macool D, Kim JH, Johnston M.** 2004. Regulatory network connecting two glucose signal transduction pathways in Saccharomyces cerevisiae. Eukaryot. Cell **3:**221–231. http://dx.doi.org/10.1128/EC.3.1.221-231.2004.
77. **Lim MK, Siew WL, Zhao J, Tay YC, Ang E, Lehming N.** 2011. Galactose induction of the GAL1 gene requires conditional degradation of the Mig2 repressor. Biochem. J. **435:**641–649. http://dx.doi.org/10.1042/BJ20102034.
78. **Westholm JO, Nordberg N, Muren E, Ameur A, Komorowski J, Ronne H.** 2008. Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. BMC Genomics **9:**601. http://dx.doi.org/10.1186/1471-2164-9-601.
79. **Berkey CD, Vyas VK, Carlson M.** 2004. Nrg1 and Nrg2 transcriptional repressors are differently regulated in response to carbon source. Eukaryot. Cell **3:**311–317. http://dx.doi.org/10.1128/EC.3.2.311-317.2004.
80. **Kuchin S, Vyas VK, Carlson M.** 2002. Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth, and diploid pseudohyphal differentiation. Mol. Cell. Biol. **22:**3994–4000. http://dx.doi.org/10.1128/MCB.22.12.3994-4000.2002.
81. **Penalva MA, Tilburn J, Bignell E, Arst HN, Jr.** 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol. **16:**291–300. http://dx.doi.org/10.1016/j.tim.2008.03.006.
82. **Young ET, Zhang C, Shokat KM, Parua PK, Braun KA.** 2012. The AMP-activated protein kinase Snf1 regulates transcription factor binding, RNA polymerase II activity, and mRNA stability of glucose-repressed genes in Saccharomyces cerevisiae. J. Biol. Chem. **287:**29021–29034. http://dx.doi.org/10.1074/jbc.M112.380147.
83. **Rodriguez-Navarro A, Benito B.** 2010. Sodium or potassium efflux ATPase a fungal, bryophyte, and protozoal ATPase. Biochim. Biophys. Acta **1798:**1841–1853. http://dx.doi.org/10.1016/j.bbamem.2010.07.009.
84. **Fraile-Escanciano A, Garciadeblas B, Rodriguez-Navarro A, Benito B.** 2009. Role of ENA ATPase in Na(+) efflux at high pH in bryophytes. Plant Mol. Biol. **71:**599–608. http://dx.doi.org/10.1007/s11103-009-9543-5.
85. **Zhou X, O'Shea EK.** 2011. Integrated approaches reveal determinants of genome-wide binding and function of the transcription factor Pho4. Mol. Cell **42:**826–836. http://dx.doi.org/10.1016/j.molcel.2011.05.025.
86. **Gonzalez A, Casado C, Petrezselyova S, Ruiz A, Arino J.** 2013. Molecular analysis of a conditional hal3 vhs3 yeast mutant links potassium homeostasis with flocculation and invasiveness. Fungal Genet. Biol. **53:**1–9. http://dx.doi.org/10.1016/j.fgb.2013.02.007.
87. **Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD.** 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:**115–132. http://dx.doi.org/10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2.



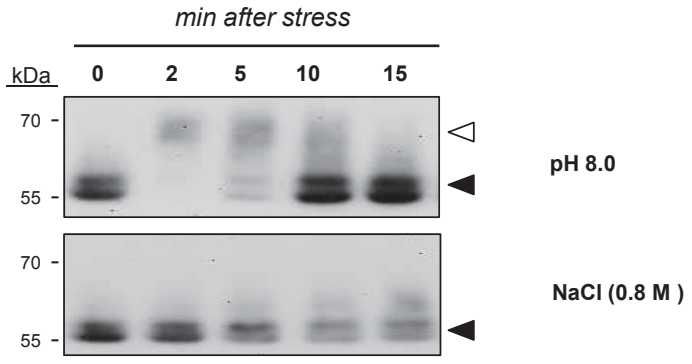
Supplemental Figure 1. **Effect of the *pho84* mutation on Pho89 expression under low Pi or high pH stress**. DBY476 wild type strain (WT) the *pho84* were transformed with pMM17-PHO89 plasmid carrying the *PHO89* gene fused with the 3x-HA epitope. Cells were grown in synthetic high P_i medium (10 mM potassium phosphate) to exponential phase (OD 0.6) and subjected to high pH or low phosphate stresses for the indicated times. Proteins (30 µg) were resolved by SDS-PAGE (10% gels) and electroblotted on PVDF filters (Immobilon-P, Millipore). Pho89-HA was detected by the use of anti-HA antibody as described in Materials and Methods.



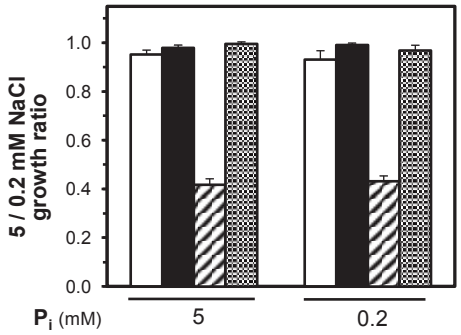
Supplemental Figure 2. **High pH stress induces transient phosphorylation and nuclear-cytoplasmic shift and Mig1.** A) A chromosomal copy of Mig1 including a C-terminal 3xHA epitope tag was introduced in wild type BY4741 cells and in its *snf1* derivative. After exposing cells to pH 8.0 for the indicated times, extracts were prepared and subjected to SDS-PAGE (8% polyacrylamide gels), prior treatment with alkaline phosphatase in the absence (-) or the presence (+, to prevent the action of the phosphatase) of 50 mM EDTA. Immunoblots were performed using anti-HA antibodies. Empty triangles denote slower (more phosphorylated) species. B) Strain SP048, containing chromosomally encoded C-terminal fusions of GFP with Mig1, were shifted to pH 8.0 and the localization of the repressor followed by fluorescence confocal microscopy. Nuclei were stained with DAPI to illustrate nuclear co-localization of the GFP and DAPI signals (merged).



Supplemental Figure 3. Wild type strain DBY746 was transformed with pPHO89-LacZ, pKC201 (carrying the entire *ENA1* promoter fused to LacZ) or pAMS366 (in which LacZ is driven by a 4x-CDRE tandem). Cells were subjected to high pH stress for 90 min (pH 8.0, closed bars), 0.4 M NaCl for 60 min (stripped bars) or 0.8 M NaCl for 90 min (dotted bars), prior determination of β-galactosidase activity. Data correspond to the mean ± SEM for 6 determinations.



Supplemental Figure 4. A chromosomal copy of Mig2 including a C-terminal 3x-HA epitope tag was introduced in wild type BY4741 cells. After exposing cells to pH 8.0 or 0.8 M NaCl for the indicated times, extracts were prepared and subjected to 8% SDS-PAGE. Immunoblots were performed using anti-HA antibodies. The empty triangle denotes slow migrating (phosphorylated) species



Supplemental Figure 5. Strains ONA1 (*pho84,* empty bars), RH16.6 (*ena1*, closed bars), ASC17 (*ena1 pho84*, stripped bars), and ASC30 (*ena1 pho89*, crossed bars) were grown in liquid media (adjusted to pH 7.2) containing the indicated concentrations of P_i and either 0.2 or 5 mM NaCl. The OD_{650} was determined after 47 h (due to the slow growth of the strains) and the 5 mM / 0.2 mM NaCl absorbance ratio calculated. Data are mean ± SEM from 5 to 9 experiments.