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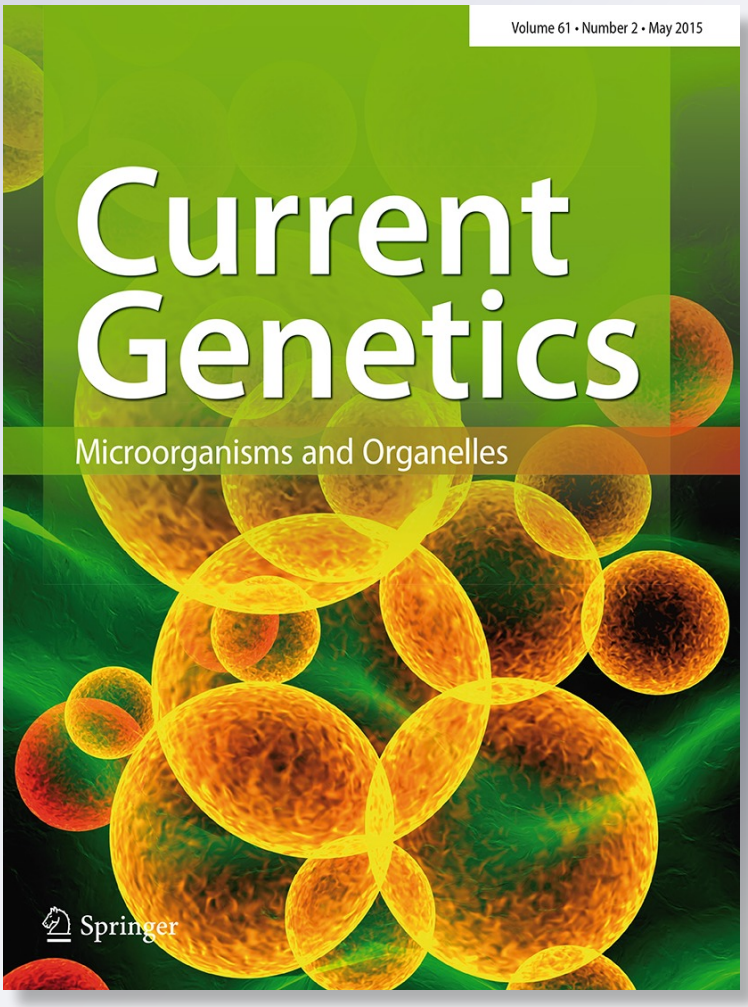

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RESEARCH ARTICLE

Dissection of the PHO pathway in *Schizosaccharomyces pombe* using epistasis and the alternate repressor adenine

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Abstract In *Saccharomyces cerevisiae*, intracellular phosphate levels are maintained by the PHO pathway, activation of which is assayed by increased phosphatase activity. The PHO pathway of *Schizosaccharomyces pombe* upregulates phosphatase activity (encoded by *pho1*$^+$) during low extracellular phosphate levels, but the underlying mechanism is poorly understood. We utilized an alternate repressor of *pho1*$^+$ expression (adenine supplementation) along with epistasis analysis to develop a model of how *S. pombe* PHO pathway components interact. Analyzing Pho1 activity in *S. pombe* PHO pathway deletion mutants during adenine starvation, we observed most mutants with a phosphatase defect in phosphate starvation also had a defect in adenine starvation. Pho7, a transcription factor in the PHO pathway, is necessary for an adenine starvation-mediated increase in Pho1 activity. Comparing adenine starvation to phosphate starvation, there are differences in the degree to which individual mutants regulate the two responses. Through epistasis studies, we identified two positive regulatory arms and one repressive arm of the PHO pathway. PKA activation is a positive regulator of Pho1 activity under both environmental conditions and is critical for transducing adenine concentrations in the cell. The synthesis of IP_7 also appears critical for the induction of Pho1 activity during adenine starvation, but IP_7 is not critical during phosphate starvation, which differs from *S. cerevisiae*. Finally, Csk1 is critical for repression of *pho1*$^+$ expression during phosphate starvation. We believe all of these regulatory arms converge to increase transcription of *pho1*$^+$ and some of the regulation acts through *pho7*$^+$.

Keywords Phosphate starvation · Inositol polyphosphates · Protein kinase A · Pho1 · Pho7 · Csk1

Introduction

Proper nutrient sensing is required for cell survival. Therefore, organisms have evolved sensing mechanisms for essential nutrients, such as inorganic phosphate, which is a ubiquitous component of cellular processes (Karthikeyan et al. 2007; Wilson et al. 2013). In the yeast *Saccharomyces cerevisiae*, cellular homeostasis of intracellular phosphate levels is maintained by the phosphate signal transduction pathway (PHO pathway) (Wykoff and O'Shea 2001). When subjected to low extracellular phosphate concentrations, the PHO pathway is activated. This activation leads to an increase in high-affinity phosphate uptake and to the secretion of phosphatases, which scavenge inorganic phosphate from extracellular organic phosphate-containing compounds (Henry et al. 2011; Kerwin and Wykoff 2009; Secco et al. 2012). The PHO pathway of *S. cerevisiae* is a relatively simple system of positive and negative regulatory components (Lenburg and O'Shea 1996). Upstream in the PHO pathway of *S. cerevisiae* is a cyclin–cyclin dependent kinase (CDK) complex regulated primarily by a metabolite, inositol pyrophosphate (IP_7) (Lee et al. 2008). The cause of increased intracellular IP_7 during external phosphate starvation is unknown, but IP_7 is the most upstream

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metabolite currently identified and regulates the CDK complex through allostery (Lee et al. 2007, 2008).

The PHO pathway in *Schizosaccharomyces pombe* also upregulates acid phosphatase expression in response to low extracellular phosphate levels (Elliott et al. 1986; Schweingruber et al. 1992). However, the mechanism by which phosphate starvation increases expression of the acid phosphatase and the phosphate transporter is much less characterized in *S. pombe* relative to *S. cerevisiae* (Carter-O'Connell et al. 2012; Henry et al. 2011). We have identified a number of genes that appear to regulate the activity of Pho7, as a *pho7*Δ strain is epistatic to the known regulatory mutants in the PHO pathway (Henry et al. 2011). Recently, it was demonstrated that a long non-coding RNA is transcribed at the $pho1^+$ locus during high external phosphate conditions, leading to the recruitment of RNAi machinery and repression of $pho1^+$ through methylation (H3K9me2) (Shah et al. 2014), although how the signal transduction pathway and this recruitment mechanism interact is currently unknown.

Cell growth is also dependent on the regulation of de novo synthesis of nucleotides, nucleotide recycling, and degradation of macromolecules that contain nucleotides. Nucleotide synthesis, such as adenine biosynthesis, is an energy-intensive process and is highly regulated (Ljungdahl and Daignan-Fornier 2012). Work in *S. cerevisiae* and *S. pombe* have demonstrated a coupling of adenine synthesis with a phosphate starvation response (Gauthier et al. 2008; Schweingruber et al. 1992). The signaling factors that control adenine biosynthesis in *S. cerevisiae* include the transcription factors Pho2, Bas1, Gcn4, and certain metabolites, although many mechanistic details remain to be elucidated (Bhoite et al. 2002; Joo et al. 2009; Pinson et al. 2009; Rebora et al. 2005). In contrast, the signaling pathways regulating adenine biosynthesis in *S. pombe* are unknown.

In *S. cerevisiae*, low adenine concentrations in growth medium affect the expression of PHO regulon genes (Gauthier et al. 2008). Expression of the *S. cerevisiae* high-affinity phosphate transporter, *PHO84*, increased ~3-fold in medium limited for adenine relative to adenine-replete conditions, even with excess inorganic phosphate present. Since an increase in *ScPHO84* expression is considered an indicator of PHO pathway activation in *S. cerevisiae*, the low adenine condition activates the PHO pathway (Gauthier et al. 2008). The mechanism behind this cross-regulation in *S. cerevisiae* is unknown. Despite *S. pombe* and *S. cerevisiae* being very distantly related (Wapinski et al. 2007), *S. pombe* also exhibits PHO pathway activation in low adenine conditions with greater than a threefold increase in Pho1 phosphatase activity, even when phosphate is not limiting (Schweingruber et al. 1992). Through the use of adenine repression of the $pho1^+$ gene, we conducted epistatic analysis of previously identified PHO pathway components. Our findings allowed us to develop a model of how *S. pombe* PHO pathway components interact and provide an initial understanding of the mechanisms underlying the cross-regulation of the *S. pombe* PHO pathway by adenine starvation.

Materials and methods

Growth conditions and strains

S. pombe cells were maintained in previously described YES and EMM media (Forsburg and Rhind 2006). Wild-type strains $h-$ (DP1) and $h+$ (DP2) were derived between 972 and 975 and generously provided by D. Moazed. Strains mutant in $csg1^+$, $cgs2^+$, or $gpa2^+$ were generously provided by Charles Hoffman. The mutant strains should accumulate cAMP and they additionally contained suppressor mutations allowing for them to be backcrossed to wild-type or *pho7*Δ strains. After backcrossing, spores were plated on EMM medium lacking amino acids (to select for prototrophy) and the mutant phenotypes were identified by poor viability in stationary phase and by the inability to mate after backcrossing. Multiple isolates were examined. Deletion strains were generated through standard replacement of a gene of interest with a marker for G-418 or nourseothricin resistance (Henry et al. 2011), and mating types for strains were determined by PCR (Forsburg and Rhind 2006). The complete list of strains can be found in Supplemental Table 1. All adenine auxotrophic *S. pombe* strains ($ade6^-$) were backcrossed into a wild-type $ade6^+$ background to enable the deletion strains to be tested in low adenine conditions (ade^- mutants are defective in the induction of $pho1^+$). *S. pombe* strains were tested for phosphatase activity using two modified mediums: 90 % SD-10 % EMM (with and without phosphate) and YE and YE5S (which is 5× the standard SP addition) (Forsburg and Rhind 2006; Henry et al. 2011). Formulations of all modified mediums are provided in Supplemental Table 2. For solid plates, 2 % Bacto-agar (Difco) was included.

PNPP assay

To test for phosphatase activity in high and low adenine conditions, strains were grown in YE5S at 30 °C to an optical density of 0.15–0.3 at 600 nm (OD_{600}) (mid-logarithmic phase). Cultures were washed with sterile water, and inoculated into 5 mL of YE5S or YE-ade medium at a low density and grown for 10 h at 30 °C. Cells were centrifuged, suspended in water, and assayed for phosphatase activity for 10 min at pH 4 as described previously (Kerwin and Wykoff 2009). PNPP (ρ-nitrophenyl phosphate) hydrolysis

was measured at OD_{400}. The OD_{400}/OD_{600} ratio for each reaction was calculated, accounting for dilutions.

Phosphatase response to high and low phosphate conditions was tested by growing strains in 90 % SD-10 % EMM with phosphate at 30 °C to an OD_{600} of 0.15–0.3. Cultures were harvested, washed, and resuspended in sterile water to an OD_{600} of approximately 0.1. Cells were inoculated into 90 % SD-10 % EMM medium with or without phosphate at a low density and grown for 4 h at 30 °C. Cells were harvested and phosphatase activity assayed as described above.

Epistasis

To observe the phenotype of double mutants, we crossed single deletion strains to create *S. pombe* strains containing two gene deletions in an *ade6*$^+$ genotype. Strains of opposite mating type, each containing one gene deletion, were crossed on ME solid medium (Forsburg and Rhind 2006). Spores were chosen for the appropriate antibiotic resistance. PCR was used to confirm the appropriate gene deletions and check mating type.

Time course *of pho1*$^+$ *and pho84*$^+$ transcript abundance

Wild-type strains were grown in YE5S medium at 30 °C to OD_{600} of 0.2–0.5. Cells were pelleted by centrifugation, washed three times, transferred to YE-ade medium, and grown at 30 °C for 10 h. Quantitative reverse-transcription PCR (RT-qPCR) was used to measure the amount of *pho84*$^+$, *pho1*$^+$, and *act1*$^+$ transcript at the selected time points during adenine starvation. The cells harvested during the time course were stored at −80 °C until the time of RNA extraction.

RT-qPCR

RNA was purified by an acid phenol protocol (Kerwin and Wykoff 2009) and quantified with a Nanodrop 2000. 1 µg of RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad), followed by a 1:5 dilution. Transcripts from 3 µl of the diluted cDNA were quantified using a Bio-Rad Chromo-4 with 50 µl Sybr green I reaction mixtures. *pho84*$^+$ and *pho1*$^+$ transcript levels were normalized to *act1*$^+$ transcript, which does not change in abundance during phosphate starvation (Henry et al. 2011).

Results

To determine the responsiveness of wild-type *S. pombe* to phosphate and adenine starvation, we tested for phosphatase activity in both growth conditions (Fig. 1a). In

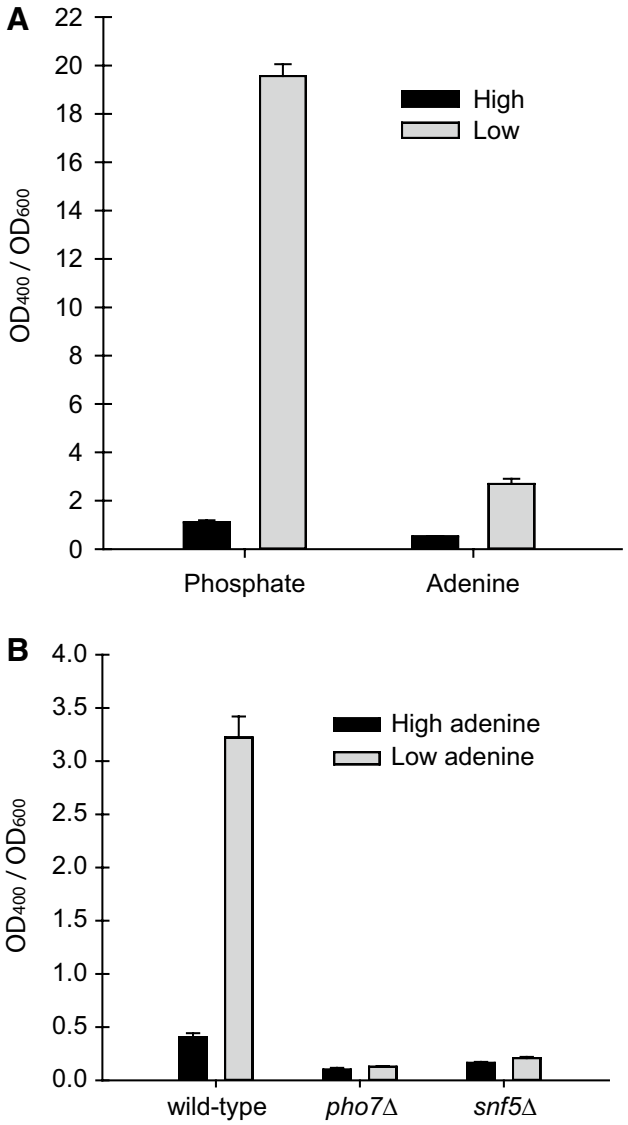

Fig. 1 **a** Phosphatase assay of wild-type *S. pombe* in high and no phosphate and high and low adenine conditions. Phosphatase activity in this figure and all following figures was measured as the amount of PNPP hydrolyzed (OD_{400}) normalized to cell density (OD_{600}) and was performed on cells grown in the same medium under the same conditions on the same day. In this figure and all future figures at least three independent cultures were grown and the *bars* are standard deviation of the mean. Repetitions on different days had identical trends. Phosphate starvation leads to a much stronger induction of phosphatase activity. **b** De-repression of Pho1 activity by adenine starvation requires both *pho7*$^+$ and *snf5*$^+$. As only adenine starvation was being measured, the scale on the *y* axis differs from (**a**)

no phosphate, there is a de-repression of Pho1 acid phosphatase activity, as seen in previous studies (Henry et al. 2011). In low adenine, there is also de-repression of *pho1*$^+$, which was observed previously (Schweingruber et al. 1992). We asked whether the two starvation conditions were additive by examining wild-type cells grown during adenine starvation, phosphate starvation, and both

starvation conditions together (Supplemental Fig. 1). It appears that adenine starvation is not additive with phosphate starvation alone, suggesting that the strong activation by phosphate starvation masks the weaker activation by adenine starvation. To determine if the increase in Pho1 activity was due to an increase in mRNA expression or an alteration of enzymatic activity, we quantified *pho1*$^+$ transcript and found that *pho1*$^+$ mRNA abundance increased during adenine starvation (Supplemental Fig. 2), suggesting that increased Pho1 activity is due to increased gene expression.

We previously identified *pho7*$^+$, a zinc-finger transcription factor, and *snf5*$^+$, a component of a chromatin remodeling complex, as the downstream effectors of the *S. pombe* PHO pathway (Carter-O'Connell et al. 2012; Henry et al. 2011) and we tested for acid phosphatase activity in *pho7*Δ and *snf5*Δ strains in high and low adenine conditions (Fig. 1b). Wild-type *S. pombe* increases phosphatase activity in low adenine conditions, and this increase is eliminated in the *pho7*Δ and *snf5*Δ mutants. Thus, *pho7*$^+$ and *snf5*$^+$ are necessary for both the adenine and phosphate starvation-mediated increase in Pho1 activity. We further hypothesized that regulators of *pho1*$^+$ during phosphate starvation would act during adenine starvation and therefore analyzed Pho1 activity in *S. pombe* PHO pathway deletion mutants during adenine starvation (Fig. 2). When comparing adenine starvation (Fig. 2) relative to phosphate starvation (Fig. 2 in Henry et al. 2011 paper) there are differences in the degree to which individual mutants regulate the two responses, suggesting cross-regulation as well as divergent regulation. For example, the *vph2*Δ and *ypa1*Δ strains have close to wild-type Pho1 phenotypes with regards to adenine starvation conditions, but are less inducible relative to wild type with regards to phosphate starvation (Henry et al. 2011).

Based on the phenotypes of the *S. pombe* mutants in high and low adenine conditions, we characterized the PHO pathway components as positive or negative regulators of *S. pombe* Pho1 activity. Through epistasis studies of the *S. pombe* gene deletions, we were able to determine the interactions between these positive and negative regulators and developed a model of the *S. pombe* PHO pathway (Fig. 3). Although our results indicate that additional factors (e.g., *ado1*$^+$) are likely involved in the PHO pathway, we were not able to conclusively place these factors within the proposed model (see Supplemental Fig. 3).

PKA activation is part of a positive regulatory arm of the PHO model (Fig. 3), and is composed of *gpa2*$^+$ and *pka1*$^+$. *gpa2*$^+$, a heterotrimeric G protein alpha-2 subunit associated with the activation of adenylate cyclase activity, and *pka1*$^+$, a cAMP-dependent protein kinase catalytic subunit, play a central role in PKA signal transduction (Gancedo 2013; Kim et al. 2013). PKA signaling is generally transduced through the *gpa2*$^+$-driven cAMP accumulation, then activation of *pka1*$^+$ (Gupta et al. 2011). Both *gpa2*$^+$ and *pka1*$^+$ are positive regulators of *pho1*$^+$ expression during phosphate starvation in *S. pombe* (Henry et al. 2011). In addition, *gpa2*Δ and *pka1*Δ *S. pombe* mutants have extremely low levels of *pho1*$^+$ expression during adenine starvation (Fig. 2). Therefore, active PKA signaling is a positive regulator of Pho1 under both environmental conditions.

Regulation of the PHO pathway in *S. cerevisiae* and *S. pombe* is known to be dependent on inositol phosphates,

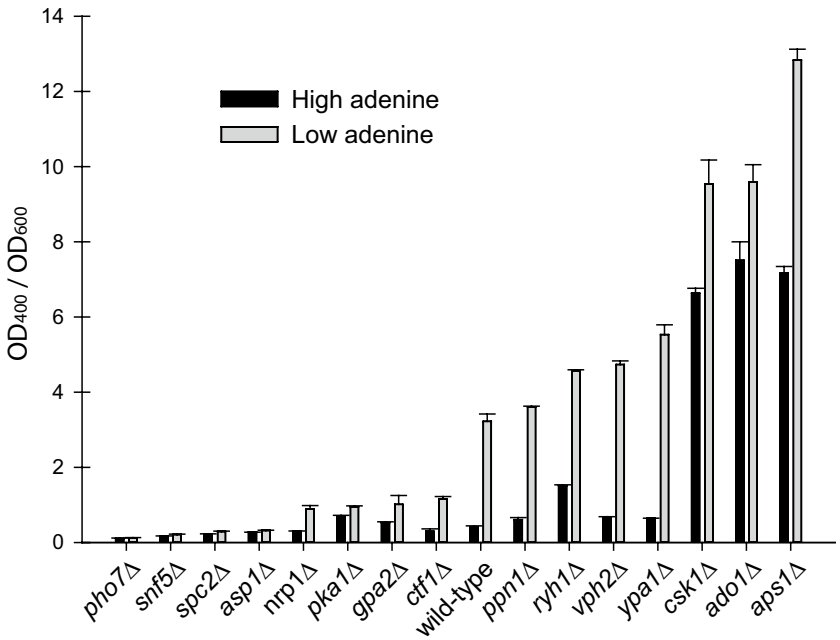

Fig. 2 Measurement of Pho1 activity in known PHO pathway mutants in adenine replete and adenine starvation conditions. These mutants were chosen based on Henry et al. (2011), backcrossed to remove auxotrophic markers, and assayed for phosphatase activity in response to adenine starvation. The mutants are sorted according to overall Pho1 activity in low adenine conditions

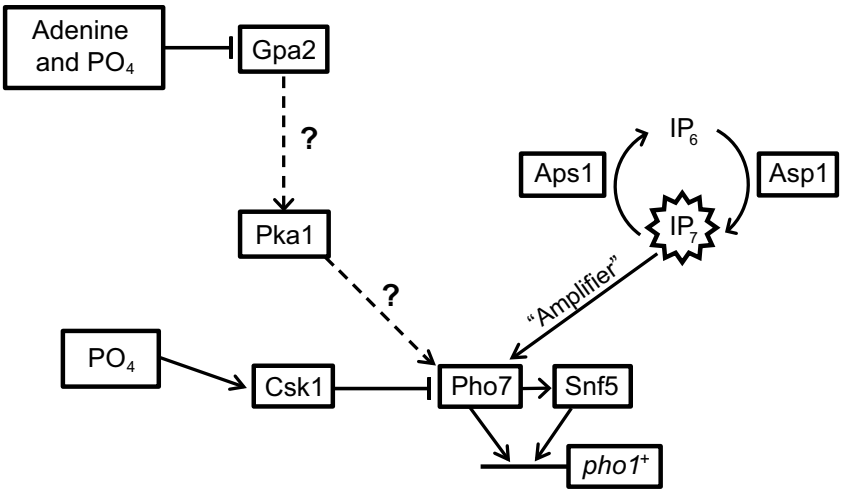

Fig. 3 Model of regulation of *pho1*$^+$. Positive and negative regulation is indicated by *arrows*. It is unclear how direct any of the actions are, but the IP_7 arm appears to act as an amplifier and not a sensor per se. The PKA arm appears more important during adenine starvation and Csk1 appears more important for repression during high phosphate conditions. We assume that the zinc finger protein, Pho7, activates chromatin remodeling (of which Snf5 is a member), but that in most cases both Pho7 and Snf5 are required for Pho1 expression. There is no direct evidence that the PKA pathway acts directly on Pho7, but we hypothesize that Pho7 phosphorylation status regulates activity. Additionally, it is appealing to expect that cAMP activates PKA activity in this regulatory pathway, but our results demonstrate that elevated cAMP levels alone do not lead to increased *pho1*$^+$ expression

such as IP_7 (Henry et al. 2011; Lee et al. 2007). IP_7 is synthesized from IP_6 by the actions of *asp1*$^+$, an IP_6 kinase, and degraded by *aps1*$^+$, an IP_7 phosphatase (Mulugu et al. 2007). The *asp1*Δ mutant has low Pho1 activity regardless of adenine concentration and the *aps1*Δ mutant has constitutive Pho1 activity (Fig. 2). Therefore, IP_7 is acting as a positive regulator of *pho1*$^+$ expression during adenine starvation. Previous work has shown that deletion of *asp1*$^+$ in *S. pombe* does not produce an obvious phenotype in phosphate starvation conditions based on a phosphatase plate assay (Henry et al. 2011). To determine whether the *asp1*Δ mutant had a divergent phenotype between phosphate and adenine starvation, we examined Pho1 activity under the replete and starvation conditions (Fig. 4). Interestingly, even though IP_7 is required for both responses as indicated by the *aps1*Δ strain being relatively constitutive under both conditions (Fig. 4), the differing phenotypes of the *asp1*Δ strain in both conditions suggests the synthesis of IP_7 is critical for the induction of Pho1 activity during adenine starvation, but not during phosphate starvation (Fig. 4). Perhaps because phosphate starvation results in a strong induction relative to adenine starvation, the requirement for IP_7 synthesis is circumvented by other unknown mechanisms.

csk1$^+$ is a CDK activating kinase which was identified as a negative regulator of *pho1*$^+$ expression in *S. pombe* during phosphate starvation (Henry et al. 2011). The *csk1*Δ mutant has a high level of phosphatase activity independent of adenine concentrations (Figs. 2, 5a). Interestingly, the *csk1*Δ strain in high and no phosphate expresses at

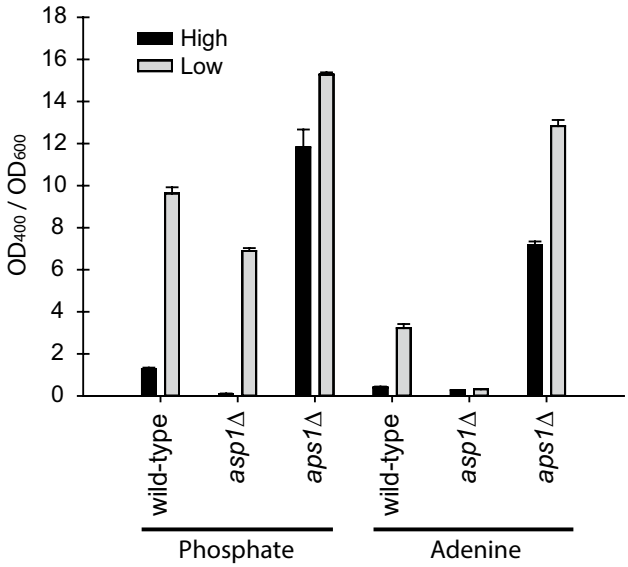

Fig. 4 Measurement of phosphatase activity in wild-type, the *asp1*Δ strain defective in IP_7 synthesis, and the *aps1*Δ strain defective in IP_7 degradation. The strains were assayed for response to both phosphate and adenine starvation

the same level as a wild-type strain in no phosphate conditions (Henry et al. 2011), but expresses twice the level of wild-type adenine starved cells (compare the wild-type and *csk1*Δ strains in Fig. 5a). This result suggests that loss of repression by Csk1 mimics phosphate starvation. Because most experiments with the *csk1*Δ strain demonstrate a slight repressibility of Pho1 activity by adenine, we

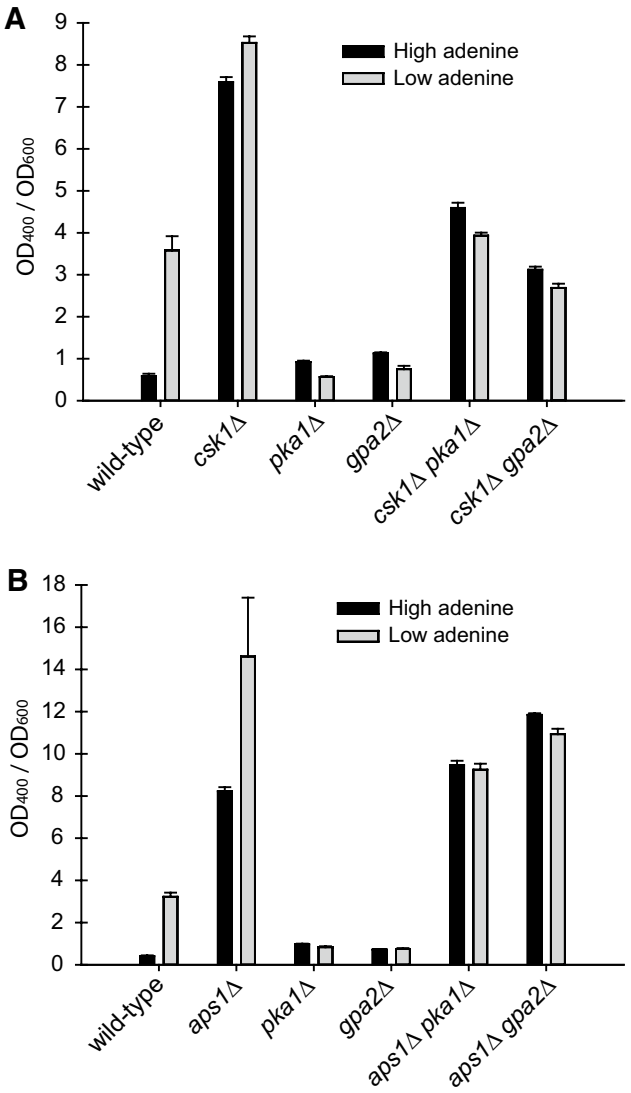

Fig. 5 **a** Epistasis with *csk1*$^+$ signaling and PKA signaling mutants. Phosphatase activity was measured in mutants lacking *csk1*$^+$, PKA signaling components, or both. **b** Epistasis with the *aps1Δ* strain defective in IP_7 phosphatase activity and PKA signaling. Pho1 phosphatase activity was measured in mutants lacking *aps1*$^+$, PKA signaling components, or both

surmise that adenine sensing does not primarily act through *csk1*$^+$ and acts through *csk1*$^+$-independent pathways.

To assess the interactions of *csk1*$^+$ with PKA signaling, we assayed the phosphatase activity of *csk1*Δ *pka1*Δ and *csk1*Δ *gpa2*Δ mutants (Fig. 5a). Loss of PKA signaling did not eliminate the high level of *pho1*$^+$ activity in the *csk1*Δ mutant but reduced phosphatase activity by half. The constitutive phenotype of *csk1*Δ *pka1*Δ and the *csk1*Δ *gpa2*Δ strains suggest activating signals of *pka1*$^+$ act independently from the repressing effects of *csk1*$^+$. The phenotypes of *csk1*Δ *gpa2*Δ and *csk1*Δ *pka1*Δ mutants support a model in which Gpa2 and Pka1 are acting together during adenine starvation and the repressing effects of Csk1 do not act through Pka1.

To determine the effect of IP_7 levels on PKA signaling, we analyzed Pho1 activity of the *aps1*Δ *pka1*Δ and *aps1*Δ *gpa2*Δ strains. Double mutants of the components of the PKA signaling pathway and the IP_6 kinase, *asp1*$^+$, were not analyzed, as the Pho1 phosphatase phenotypes of both are uninducible and epistasis analysis is uninformative. Deletion of the IP_7 phosphatase *aps1*$^+$ produced an increase in Pho1 activity even with the loss of PKA signaling (Fig. 5b), suggesting that PKA signaling is not required for IP_7-dependent *pho1*$^+$ expression. Neither the *aps1*Δ *gpa2*Δ nor the *aps1*Δ *pka1*Δ mutant exhibited an increase in phosphatase activity in low adenine relative to high adenine. This phenotype suggests that the elevated IP_7 levels increase basal *pho1*$^+$ expression, but to de-repress the PHO pathway during adenine starvation, activation of PKA signaling is required.

We also investigated the interactions of Csk1 with IP_7. The phenotype of the *aps1*Δ *csk1*Δ strain is constitutive, with Pho1 levels greater than that of *aps1*Δ or *csk1*Δ alone (Fig. 6a), indicating that the removal of Csk1, combined with IP_7 accumulation in the Aps1 phosphatase deletion, strongly de-represses *pho1*$^+$ activity. The additive phenotype of *aps1*Δ *csk1*Δ suggests that Csk1 and IP_7 serve distinct roles in *pho1*$^+$ expression regulation and act independent of one another. Analysis of Csk1 with the IP_6 kinase Asp1 also supports the establishment of separate roles. The Pho1 phosphatase activity of the *asp1*Δ *csk1*Δ mutant (Fig. 6b) exhibits a phenotype intermediate to *asp1*Δ and *csk1*Δ, suggesting that loss of IP_7 can only impact approximately half of the starvation signal. Interestingly, the phosphatase expression of the *asp1*Δ *csk1*Δ mutant was more similar to that of *asp1*Δ than *csk1*Δ, suggesting that the loss of the repressor (Csk1) is not able to efficiently release Pho1 repression if IP_7 levels are insufficient. One possible hypothesis is that Csk1 and IP_7 act through a common factor, such as Pho7, and the removal of Csk1 is insufficient to fully activate Pho7 in the absence of IP_7.

Discussion

The *S. pombe* PHO pathway utilizes both positive and negative regulators of *pho1*$^+$ expression and its pathway architecture is dramatically different from *S. cerevisiae*. The positive regulators are PKA signaling and accumulation of IP_7, with the negative regulator being Csk1. Our data suggest that IP_7 and Csk1 regulatory signals converge upon Pho7 and the SWI/SNF chromatin remodeling complex (Fig. 3). We provide evidence that the mechanism by which adenine starvation activates the PHO pathway is mediated

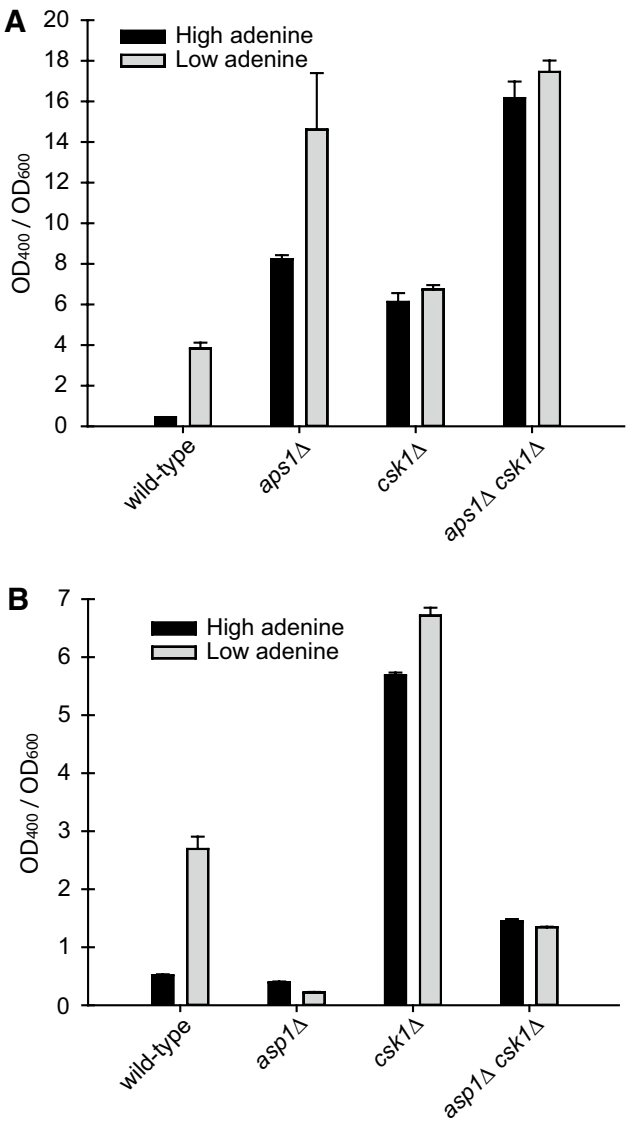

Fig. 6 **a** Epistasis in the *aps1Δ* strain defective in IP_7 phosphatase activity and the *csk1Δ*. **b** Epistasis in the *asp1Δ* strain defective in IP_6 kinase activity and the *csk1Δ*. Pho1 phosphatase activity was measured in mutants containing one or both deletions

by PKA signaling, is dependent on the availability of IP_7, and is repressed by Csk1 activity. It is worth noting that Pho1 de-repression is likely more dependent on *pho7*$^+$ than *snf5*$^+$, as accumulation of IP_7 activates *pho1*$^+$ expression when *snf5*$^+$ is deleted, but not when *pho7*$^+$ is deleted (Supplemental Fig. 4). These results suggest that pho7 is essential for the induction of *pho1*$^+$, but that the *snf5*$^+$ requirement can be circumvented by activation of other factors (presumably Pho7). *pho7*$^+$ mediates a number of starvation responses in *S. pombe*, although the regulation of *pho1*$^+$ in particular is sensitive to *pho7*$^+$ (Carter-O'Connell et al. 2012). We conclude that *pho7*$^+$ is absolutely essential for the *pho1*$^+$ de-repression and is the site of regulation by phosphate and adenine, while chromatin remodeling is subsequently required for efficient gene expression.

The PHO pathways of *S. cerevisiae* and *S. pombe* have been shown to exhibit a de-repression of *pho1*$^+$ expression during both phosphate and adenine starvation (Carter-O'Connell et al. 2012; Gauthier et al. 2008; Henry et al. 2011; Schweingruber et al. 1992). Although the evolutionary significance of this cross-regulation of the PHO pathway by adenine is unknown, we speculate that it may be a molecular "anticipatory" mechanism. As ATP is generated from both adenine and phosphate, adenine and phosphate metabolism are intimately tied to cellular metabolism (Chapman and Atkinson 1977; Lagunas 1986). An adenine deficiency may indicate that a cell is also likely to be phosphate deficient, preemptively activating the PHO pathway. Alternatively, an adenine deficiency may indicate that phosphate will be required in the near future to manufacture ATP from newly manufactured adenosine, also validating the activation of the PHO pathway.

Our data suggest that the mechanism by which adenine starvation activates the *S. pombe* PHO pathway involves PKA signaling and is also IP_7 dependent. The PHO pathway of *S. cerevisiae* utilizes IP_7 to regulate expression of PHO genes through non-covalent interactions with the Pho80/Pho85/Pho81 CDK complex (Lee et al. 2008). Although the PHO pathways of both species converge upon common orthologous gene targets, *S. pombe* appears to have evolved most of the regulatory interactions of the PHO pathway independently from that of *S. cerevisiae*, as there is no CDK complex present in *S. pombe* that regulates PHO genes (Henry et al. 2011; Tanaka and Okayama 2000). Although both species use IP_7 as a signaling component, the usage or "sensing" of IP_7 may have developed independently, as IP_7 is central to the phosphate starvation response in *S. cerevisiae*, but IP_7 signaling is somewhat disposable in *S. pombe.* In support of this is the observation that an *asp1*Δ strain in *S. cerevisiae* (gene is called *VIP1*) is uninducible for phosphatase activity (Lee et al. 2007), whereas the same deletion in *S. pombe* does not have an observable phenotype (Fig. 4). However, IP_7 is important in signaling in *S. pombe*, just not as central to the PHO regulatory pathway. Similarly, the PKA pathway appears to play a more peripheral role in the PHO pathway in *S. cerevisiae*, but a more central role in *S. pombe.*

S. cerevisiae has interactions between PKA signaling and the PHO pathway. After phosphate starvation, phosphate addition rapidly activates PKA activity independent of cAMP production and independent of phosphate transport through the transceptor Pho84 (Giots et al. 2003; Popova et al. 2010). However, others have recently indicated detectable cAMP increases upon phosphate feeding (Conway et al. 2012). Regardless, studies indicate that lower cAMP levels are correlated with multiple

nutrient starvation conditions in *S. cerevisiae*, suggesting that cAMP levels should be lower during phosphate starvation (Conway et al. 2012; Ma et al. 1997; Markwardt et al. 1995). PKA signaling does not appear to be a core signaling component of the PHO pathway in *S. cerevisiae*, but is a core requirement for the *S. pombe* PHO pathway. To determine if elevated cAMP levels activate the expression of *pho1*$^+$, we examined three mutants (*cgs1, cgs2,* and an activated *gpa2* allele*)* that were likely to elevate cAMP levels in the cell and determined those mutants effect on Pho1 activity (Supplemental Fig. 5). Surprisingly, all three were able to increase *pho1*$^+$ expression during adenine starvation, indicating that while PKA (and *gpa2*$^+$) activity is likely required for induction, elevated cAMP levels alone are not sufficient to increase expression of *pho1*$^+$. Future studies are required to determine if there is indeed a cAMP signaling difference between the two species or if this is just a complication of different experimental regimes.

Even though both adenine and phosphate starvation act either directly or indirectly through *pho7*$^+$, the mechanism by which phosphate starvation activates the *S. pombe* PHO pathway differs from that of adenine starvation. For example, depletion of IP_7 does not alter Pho1 phosphatase activity during phosphate starvation as it does in adenine starvation. Additionally, in *S. cerevisiae*, adenylate kinase (Adk1) and adenosine kinase (Ado1) have both been shown to impact purine as well as phosphate metabolism, an effect that may be acting through metabolic intermediates or Pho2, a transcription factor (Gauthier et al. 2008). Our data indicate *ado1*$^+$ signaling requires IP_7 synthesis (Supplemental Fig. 3). Further dissection of the adenine/phosphate cross-regulation requires knowledge of the adenine starvation responsive transcription factors, which we are currently attempting to identify in *S. pombe*.

Adenine starvation is a tool that can be utilized in future studies of the *S. pombe* PHO pathway. We propose a model of the regulation of PHO promoters based on epistatic interactions and de-repression by two different environmental signals. This model facilitates the comparison of phosphate metabolism between *S. pombe* and other yeast species. Additionally, this model is a starting point for the clarification of mechanistic details, such as Pho7 phosphorylation, and determination of whether Csk1 is acting, directly or indirectly, on Pho7 or Snf5. By better understanding the evolutionary changes in the metabolic pathways of single-celled eukaryotes, we may better understand the interactions of metabolic pathways in more complex eukaryotes.

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