

Positive Feedback Regulates Switching of Phosphate Transporters in *S. cerevisiae*

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DOI 10.1016/j.molcel.2007.07.022

SUMMARY

The regulation of transporters by nutrient-responsive signaling pathways allows cells to tailor nutrient uptake to environmental conditions. We investigated the role of feedback generated by transporter regulation in the budding yeast phosphate-responsive signal transduction (PHO) pathway. Cells starved for phosphate activate feedback loops that regulate high- and low-affinity phosphate transport. We determined that positive feedback is generated by PHO pathway-dependent upregulation of Spl2, a negative regulator of low-affinity phosphate uptake. The interplay of positive and negative feedback loops leads to bistability in phosphate transporter usage—individual cells express predominantly either low- or high-affinity transporters, both of which can yield similar phosphate uptake capacity. Cells lacking the high-affinity transporter, and associated negative feedback, exhibit phenotypes that arise from hysteresis due to unopposed positive feedback. In wild-type cells, population heterogeneity generated by feedback loops may provide a strategy for anticipating changes in environmental phosphate levels.

INTRODUCTION

The availability of nutrients often limits the growth of microbes (Abelson, 1999; Gray et al., 2004). Organisms employ nutrient-responsive regulatory networks to monitor nutrient levels and adjust cellular processes accordingly (Gray et al., 2004; Oshima, 1997). Nutrient homeostasis, the ability to maintain a relatively constant internal level of the limiting nutrient, is achieved by controlling the balance of nutrient uptake and utilization.

Homeostasis is a systems property, arising from interactions between components of a signaling network.

Several networks responsible for nutrient sensing in budding yeast share a common overall architecture, but it is unclear what aspects of this network structure are important for homeostasis. Many networks are responsive to internal levels of nutrient (Barnes and Zierath, 2005; Waters and Eide, 2002; Wisnicka et al., 1997; Wykoff and O'Shea, 2001). Additionally, for most nutrients, there exist high- and low-affinity transporters that have different uptake properties (Auesukaree et al., 2003; Bun-Ya et al., 1991; Harris et al., 2001; Tomas-Cobos et al., 2004); regulation of these transporters allows nutrient uptake to be optimized over a wide range of extracellular nutrient concentrations. High-affinity transport systems are transcriptionally upregulated in response to starvation to allow uptake in low nutrient environments (Bun-Ya et al., 1991; Waters and Eide, 2002). During starvation, some low-affinity nutrient transporters are selectively downregulated, although the functional role of this downregulation and its effect on systems properties are unclear (Bird et al., 2004; Harris et al., 2001; Jensen and Culotta, 2002; Tomas-Cobos et al., 2004; Waters and Eide, 2002).

Alteration of the activity of high- or low-affinity transporters has the potential to generate feedback in nutrient homeostatic systems; transporters can influence the internal level of nutrient, which in turn influences the signaling pathway, which can feed back to control the transporters. For nutrient-responsive signaling pathways that are activated by decreases in nutrient availability, upregulation of transporters could lead to increased nutrient uptake and negative feedback or the tendency to reduce the activity of the signal transduction pathway. Conversely, downregulation of transporters can generate positive feedback by reducing internal levels of nutrient, leading to further activation of the signaling pathway. In other systems, feedback has been demonstrated to confer system properties such as increased sensitivity, dampening of noise or oscillations, hysteresis, multistability, and a rapid approach to steady-state values (Acar et al., 2005; Brandman et al., 2005; Ferrell, 2002).

The *S. cerevisiae* phosphate-responsive signaling pathway (PHO pathway) is thought to monitor cytoplasmic levels of inorganic phosphate, allowing cells to sense and respond to changes in phosphate availability through

transcriptional control of genes required for uptake and scavenging of phosphate and phosphate containing compounds, and mobilization of internal phosphate stores (Auesukaree et al., 2004; Lenburg and O'Shea, 1996; Wykoff and O'Shea, 2001). There are five known phosphate transporters: three uptake phosphate with low-affinity (Pho87, Pho90, and Pho91), one is the major high-affinity phosphate transporter (Pho84), and another is utilized under specialized conditions (Pho89) (Persson et al., 1998). Pho89 and Pho84 are transcriptionally upregulated during phosphate limitation (Persson et al., 1998; Bun-Ya et al., 1991), and this upregulation requires Pho4, a transcription factor regulated by a kinase complex composed of the cyclin Pho80, cyclin-dependent kinase (CDK) Pho85, and CDK inhibitor Pho81 (Oshima, 1997; Schneider et al., 1994). In high-phosphate conditions, this complex phosphorylates Pho4, causing it to be localized to the cytoplasm (O'Neill et al., 1996). When cells are limited for phosphate, Pho81 inhibits the kinase complex and unphosphorylated Pho4 accumulates in the nucleus where it activates transcription of phosphate-responsive genes (O'Neill et al., 1996; Schneider et al., 1994). When the extracellular concentration of phosphate is between these two extremes, the kinase complex is partially active, leading to accumulation of a nuclear, partially phosphorylated form of Pho4 that is capable of activating transcription of a subset of phosphate-responsive genes, including the high-affinity phosphate transporter Pho84 (Springer et al., 2003).

In this study, we investigate regulation of low-affinity phosphate transport, the feedback generated by this regulation, and the effect that feedback has on systems properties of the phosphate-responsive regulatory network. We find that yeast cells regulate low-affinity transport activity in response to phosphate availability and demonstrate that induction of the phosphate-responsive gene *SPL2* is necessary and sufficient for PHO pathway-dependent downregulation of low-affinity transport. This downregulation creates positive feedback that generates bistability, causing individual cells to express either low- or high-affinity transporters. These findings provide insight into the origins of the phenotype of cells lacking the high-affinity phosphate transporter, which can now be understood to arise from hysteresis caused by positive feedback.

RESULTS

Low-Affinity Phosphate Transport Is Downregulated by the PHO Pathway

Because precedent exists for downregulation of some low-affinity transporters in response to nutrient limitation (Eide, 2003; Waters and Eide, 2002), we examined whether low-affinity phosphate transport is regulated in response to phosphate availability. Transcription of the three low-affinity proton/phosphate transporters *PHO87*, *PHO90*, and *PHO91* is not regulated in response to external phosphate availability (Auesukaree et al., 2003). To determine if low-affinity phosphate transport is regulated

posttranscriptionally, we measured phosphate uptake in a strain lacking the high-affinity transporter Pho84 (in which phosphate transport is primarily low-affinity, $K_m \sim 200 \mu\text{M}$ phosphate [Wykoff and O'Shea, 2001]), either pregrown for 24–48 hr in high phosphate conditions or grown in no-phosphate conditions (for details of growth conditions, see Figure 1A legend). When pregrown in high-phosphate conditions, *pho84Δ* cells take up phosphate with a velocity similar to that of wild-type cells. In contrast, when pregrown in phosphate limiting conditions, *pho84Δ* cells lose almost all phosphate transport, indicating that low-affinity transport is downregulated in response to phosphate limitation (Figure 1A). Inactivation of the downstream transcription factor Pho4 prevents this downregulation; the *pho84Δpho4Δ* strain displays little change in phosphate uptake in response to phosphate starvation (Figure 1A). We conclude that a functional PHO pathway is required for downregulation of low-affinity phosphate transport in response to phosphate limitation.

Phenotypes of the *pho84Δ* Strain Result from Feedback in the PHO Pathway

Deletion of *PHO84* results in constitutive activation of the PHO pathway, which can be measured by monitoring the expression of the secreted acid phosphatase Pho5 (Bun-Ya et al., 1991) (Figure 1B). *pho84Δ* strains also exhibit other phenotypes—they are unable to grow on low-phosphate medium and exhibit resistance to the toxic phosphate analog arsenate (Figure 1B) (Bun-Ya et al., 1991). Although it has been thought that these phenotypes result from a reduction in phosphate uptake due to loss of the phosphate transporter Pho84, it is unlikely that these defects arise solely from loss of Pho84 function, because unexpectedly, mutations that inactivate the PHO pathway (e.g., *pho81Δ* or *pho4Δ*; Figure 1B) suppress the growth phenotypes of the *pho84Δ* strain. Because a mutation that inactivates the PHO pathway can also prevent downregulation of low-affinity phosphate transport (Figure 1A), we speculate that positive feedback in the PHO pathway (through PHO pathway-dependent downregulation of low-affinity transporters in response to phosphate limitation) is the cause of the phenotypes associated with loss of Pho84 function. We hypothesize that, in cells lacking Pho84, activation of the PHO pathway triggers downregulation of low-affinity transport, causing cells to uptake less phosphate and further activate the PHO pathway, ultimately driving them to a state where the PHO pathway is constitutively on and transport capacity is minimal. This state results in a defect in low-phosphate growth and resistance to the toxic phosphate analog arsenate. Consistent with this model, activation of Pho4 is sufficient for downregulation of low-affinity phosphate transport; introduction of a constitutively activated allele of *PHO4* (Komeili and O'Shea, 1999) into the *pho84Δ pho81Δ* strain restores the arsenate resistance phenotype, presumably by restoring the ability to downregulate low-affinity transport and therefore uptake less arsenate (Figure 1B).

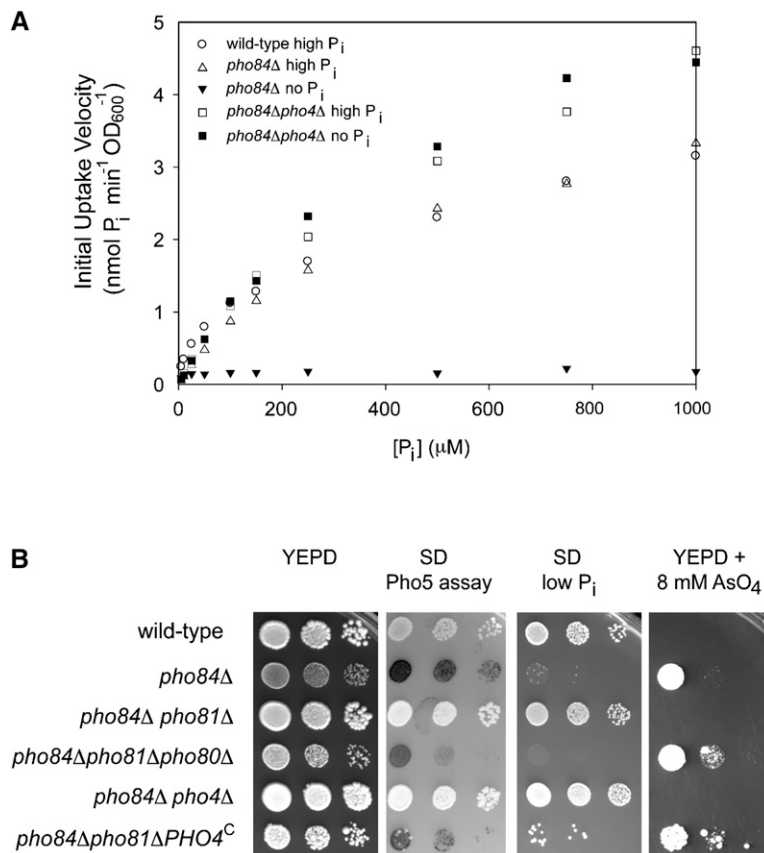


Figure 1. Phosphate Uptake and Growth of Wild-Type and Mutant Strains

(A) EY57 (wild-type), EY105 (*pho84Δ*), and EY329 (*pho84Δpho4Δ*) were grown in logarithmic phase for 24–48 hr in high-phosphate medium (SD medium containing 15 mM phosphate) and transferred to SD or SD medium lacking phosphate for 4 hr at 30°C, and ³²P uptake measurements were performed as described previously (Wykoff and O’Shea, 2001). The initial uptake velocity of wild-type cells grown in no-phosphate conditions is not displayed; the uptake velocity values approach 20 nmol phosphate min⁻¹ OD₆₀₀⁻¹. EY105 exhibited a 50% defect in phosphate uptake when inoculated from a plate and grown for 8–10 hr in SD medium (Wykoff and O’Shea, 2001), but this defect was suppressible by growth for 24–48 hr in SD medium containing 15 mM phosphate.

(B) Strains EY57, EY105, EY152 (*pho84Δ pho81Δ*), EY334 (*pho84Δpho81Δpho80Δ*), EY329, and EY1880 (*pho84Δpho81Δ PHO4^{SA1-4PA6}*) were grown overnight in SD medium, diluted to OD₆₀₀ ~0.3, and plated in 10-fold dilutions. The first three panels of photographs are of plates that were incubated for two days at 30°C. The last panel is a photograph of a plate that was incubated for 5 days at 30°C. In the second panel, the plate was overlaid with a substrate to detect Pho5 acid phosphatase activity (Bun-Ya et al., 1991).

Spl2 Is Necessary and Overexpression Is Sufficient to Downregulate Low-Affinity Phosphate Transport

Because Pho4 is a known activator of transcription (Barbaric et al., 1998), we hypothesized that Pho4 activates transcription of a gene whose protein product then downregulates low-affinity phosphate transport. To identify this gene, we inactivated 21 Pho4-dependent genes (Carroll et al., 2001; Springer et al., 2003) in a *pho84Δ* background and assayed these double-mutant strains for the ability to grow in medium containing arsenate (Figure S1 in the Supplemental Data available with this article online). We identified only one Pho4-induced gene, other than *PHO81*, that suppressed the arsenate resistance phenotype of the *pho84Δ* strain when inactivated: *SPL2* (Figure 2A). Like deletion of *PHO4* (and *PHO81*), deletion of *SPL2* in the *pho84Δ* strain restores arsenate sensitivity and low-affinity phosphate transport in low-phosphate conditions (Figure 2A). However, in contrast to the *pho84Δpho4Δ* strain, the PHO pathway is otherwise regulated appropriately in the *pho84Δspl2Δ* mutant, as evidenced by the ability to control expression of the phosphate-responsive gene *PHO5* (Figure 2A). *spl2Δ* mutants exhibit no obvious defect in *PHO5* induction (Flick and Thorer, 1998) and data not shown), explaining why *SPL2* was not identified in previous screens for mutants defective in PHO pathway regulation. These observations strongly suggest that Spl2

is required for downregulation of low-affinity phosphate transport in conditions of phosphate limitation. In support of these results, our genetic evidence indicates that Spl2 downregulates the low-affinity transporters Pho87 and Pho90 (Figure S2).

If it is true that positive feedback resulting from Spl2-dependent downregulation of low-affinity transport causes the *pho84Δ* phenotypes, deletion of *SPL2* in a *pho84Δ* strain should increase internal phosphate levels, and in turn increase Pho80-Pho85 kinase activity, in high-phosphate conditions. To test if this prediction is correct, we measured Pho80-Pho85 kinase activity in *pho84Δ* and *pho84Δspl2Δ* strains grown in high-phosphate conditions with Pho4-YFP localization as a reporter. We find that Pho80-Pho85 is inactive in the *pho84Δ* strain and is active in the *pho84Δspl2Δ* strain (Figure S3). Thus, the phenotypes of the *pho84Δ* strain are a consequence of inappropriate activation of a positive feedback loop involving Pho4-dependent activation of *SPL2*, leading to downregulation of low-affinity transport, less internal phosphate, and further PHO pathway activation. In the absence of *PHO84* induction, positive feedback causes cells to become trapped in a state where the PHO pathway is constitutively activated.

To determine whether *SPL2* expression is sufficient to downregulate low-affinity phosphate transport, we compared the phosphate uptake kinetics and gene expression

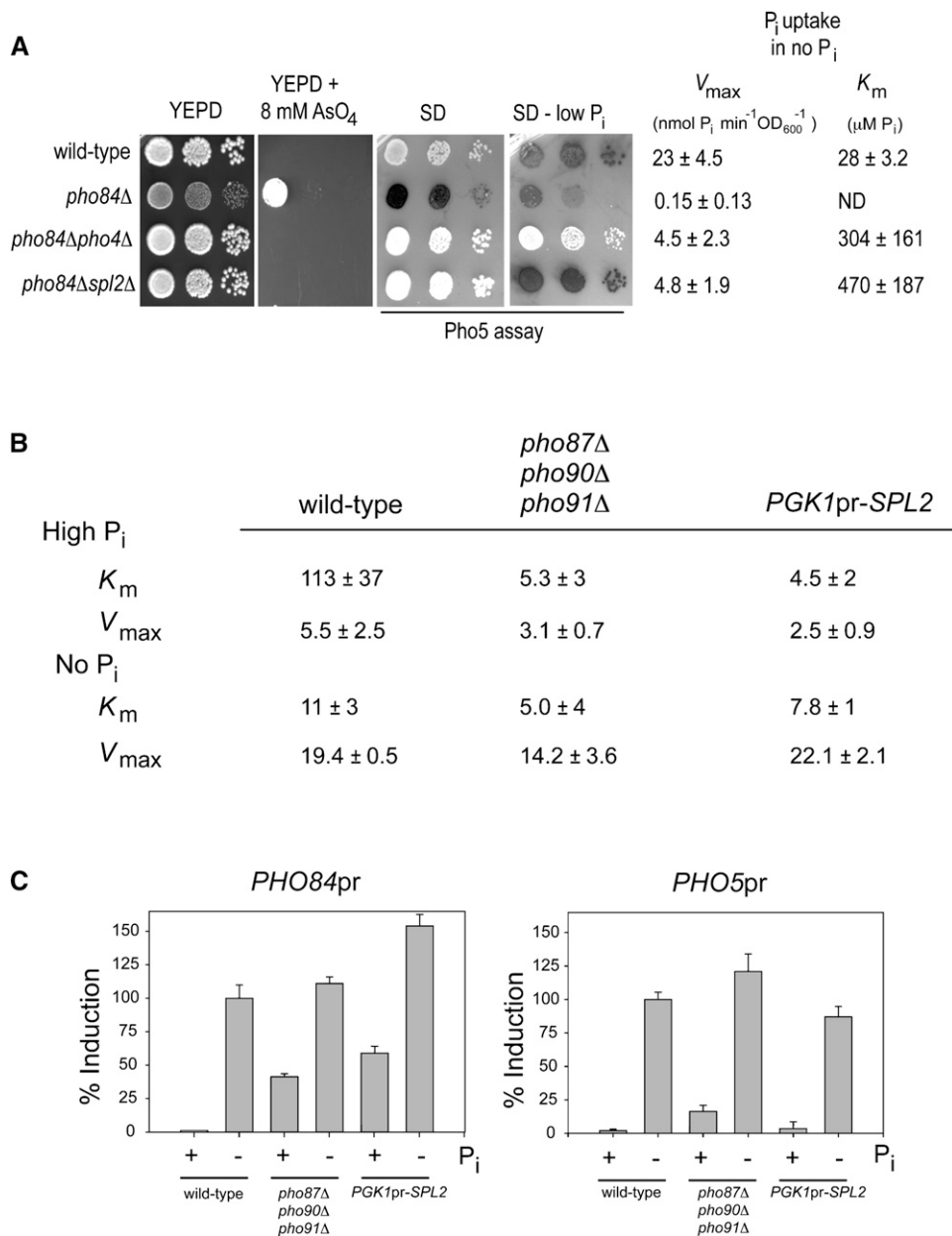


Figure 2. Spl2 Is Necessary and Overexpression Is Sufficient to Downregulate Low-Affinity Phosphate Transport

(A) Strains EY57 (wild-type), EY105 (*pho84Δ*), EY329 (*pho84Δ pho4Δ*), and EY1718 (*pho84Δ spl2Δ*) were grown and plated as described in Figure 1, except that the YEPD + 8 mM AsO_4 plate was incubated for 3.5 days at 30°C. The aggregate V_{max} and K_m were calculated from phosphate uptake data of three independently grown cultures, and the error is the standard deviation. Because there is negligible phosphate uptake in the *pho84Δ* strain, the K_m was not determined.

(B) Strains EY57 (wild-type), EY1982 (*pho87Δpho90Δpho91Δ*), and EY1959 (*PGK1pr-SPL2*) were grown in high- or no-phosphate medium for 4 hr. Phosphate uptake measurements were performed, and the aggregate kinetic constants were derived. The error is the standard deviation of three independent experiments. V_{max} and K_m values are reported in units of nmol P_i min⁻¹ OD₆₀₀⁻¹ and μ M P_i , respectively.

(C) Strains containing integrated versions of the *PHO84* and *PHO5* promoters controlling GFP expression (the *PHO84pr*-GFP reporter is integrated at the *URA3* locus so that the wild-type copy of *PHO84* is intact) were grown in high- and no-phosphate medium and assayed by flow cytometry. The fluorescence was background subtracted and normalized to total induction of wild-type cells. The errors are the standard deviation between the mean fluorescence of three independent cultures. The strains are EY2094 (*PHO5pr*-GFP), EY1981 (*pho87Δpho90Δpho91Δ PHO84pr*-GFP), EY1958 (*PGK1pr-SPL2 PHO84pr*-GFP), EY1995 (*PHO84pr*-GFP), EY2044 (*pho87Δpho90Δpho91Δ PHO5pr*-GFP), and EY2045 (*PGK1pr-SPL2 PHO5pr*-GFP).

phenotypes caused by overexpression of *SPL2* in high-phosphate conditions to those resulting from deletion of the low-affinity phosphate transporters. When *SPL2* is overexpressed (Figure S4) in high-phosphate conditions, we observe a 20-fold decline in the K_m of phosphate uptake (Figure 2B), consistent with loss of low-affinity phosphate uptake and a compensatory increase in Pho84 activity. As expected if Spl2 overexpression inactivates low-affinity transport, the K_m we observe in cells overexpressing *SPL2* is similar to the K_m observed in cells lacking the three low-affinity phosphate transporters (Pho87, Pho90, and Pho91). Inactivation of low-affinity transporters also causes changes in the activity of the PHO signaling pathway that can be quantified by monitoring expression of transcriptional reporters for the phosphate-responsive genes *PHO84* and *PHO5*. *SPL2* overexpression and deletion of low-affinity transporters induce similar levels of *PHO84* (Figure 2C, left panel) but little induction of *PHO5* (Figure 2C, right panel). The differential effect on *PHO5* and *PHO84* induction can be explained by the higher sensitivity of *PHO84* to changes in the PHO pathway; low-affinity transporter deletion only partially activates the PHO pathway, and *PHO84* is induced at a higher phosphate concentration and lower level of pathway activation than is *PHO5* (Thomas and O'Shea, 2005). We conclude that overexpression of *SPL2* (in high-phosphate conditions) phenocopies deletion of the low-affinity transporters, indicating that it is sufficient to downregulate low-affinity transport.

Spl2 Alters Systems Properties of the PHO Pathway

In high-phosphate conditions, neither *SPL2* nor *PHO84* is significantly expressed (Bun-Ya et al., 1991; Lau et al., 2000; Figure S4), and cells use low-affinity transporters to uptake phosphate into the cell. *SPL2* and *PHO84* induction in conditions of phosphate starvation causes cells to switch their complement of phosphate transporters from low to high affinity, allowing cells to uptake phosphate under conditions where it is limiting. Our previous studies revealed that in intermediate phosphate conditions (between these two extremes of phosphate replete and phosphate limiting) *PHO84* expression is induced to ~50% of maximal levels (Springer et al., 2003). This submaximal induction is at least partly a consequence of population heterogeneity (Thomas and O'Shea, 2005) that is readily observed when *PHO84* expression is monitored by flow cytometric analysis of single cells containing a transcriptional reporter in which GFP expression is under the control of the *PHO84* promoter (*PHO84pr-GFP*; integrated into the yeast genome at an ectopic locus so as to maintain a wild-type copy of *PHO84*). When cells expressing *PHO84pr-GFP* are grown in high or no phosphate, most cells exhibit either low or very high GFP expression, respectively (Thomas and O'Shea, 2005; Figure 3A). In contrast, over a range of phosphate concentrations from ~100 to 200 μM phosphate (intermediate phosphate), there are two popula-

tions of cells at steady state: one that expresses little *PHO84* and another that highly expresses *PHO84* (Figure 3A). These observations suggest that, in intermediate phosphate conditions, part of the population uses Pho84 to uptake phosphate and the remainder uses a different transport system.

To determine if the two populations of cells observed in intermediate phosphate use different transport systems to uptake phosphate, we sorted wild-type cells from intermediate phosphate conditions based on their expression of *PHO84pr-GFP* and measured phosphate uptake kinetics and Spl2 expression (Figure 3B). Cells expressing high levels of *PHO84* have a K_m consistent with high-affinity phosphate transport (~7 μM), whereas cells expressing little *PHO84* use predominantly low-affinity phosphate transporters for uptake (K_m ~150 μM). The velocity of phosphate uptake in each population grown in 150 μM phosphate is similar (1.75 ± 0.36 versus 1.38 ± 0.32 nmol phosphate min^{-1} OD_{600}^{-1} , respectively), suggesting that cells have approximately the same uptake rate but utilize different uptake systems to achieve this. As assayed by immunoblot analysis (Figure S5), cells that express low levels of *PHO84* also express little Spl2, whereas cells that induce *PHO84* express a high level of Spl2. Therefore, the two populations of cells in intermediate phosphate differ in the phosphate transporters they use to obtain phosphate from the environment.

Because positive feedback can cause bimodal expression and bistability (Ferrell, 2002) and Spl2 is postulated to be part of a positive feedback loop in the PHO pathway, we tested if bimodal expression of *PHO84* was dependent on Spl2 (Figure 3C). Cells lacking *SPL2* express *PHO84pr-GFP* in a graded fashion, suggesting that *SPL2* induction is a positive feedback (or double-negative feedback) element in the PHO pathway required for bimodal expression of *PHO84* in intermediate phosphate conditions. Although we observe a striking effect of Spl2 on *PHO84* expression, we do not find a difference in growth of wild-type and *spl2 Δ* mutant cells in high- or no-phosphate conditions (data not shown).

DISCUSSION

We have demonstrated that feedback exists in the PHO pathway that allows yeast cells to switch their complement of phosphate transporters in response to phosphate availability. In response to a decrease in internal phosphate levels, cells activate the PHO pathway, triggering two feedback elements: a negative feedback loop consisting of Pho4-dependent induction of *PHO84*, which helps to bring phosphate into the cell and inactivate the PHO pathway, and a positive feedback loop consisting of Pho4-dependent upregulation of *SPL2*, which tends to reduce phosphate uptake, leading to further pathway activation (Figure 4). Because the feedback loops are both controlled by the PHO pathway, they create mutually exclusive states in which wild-type cells either activate the PHO pathway, express high-affinity transporters, and

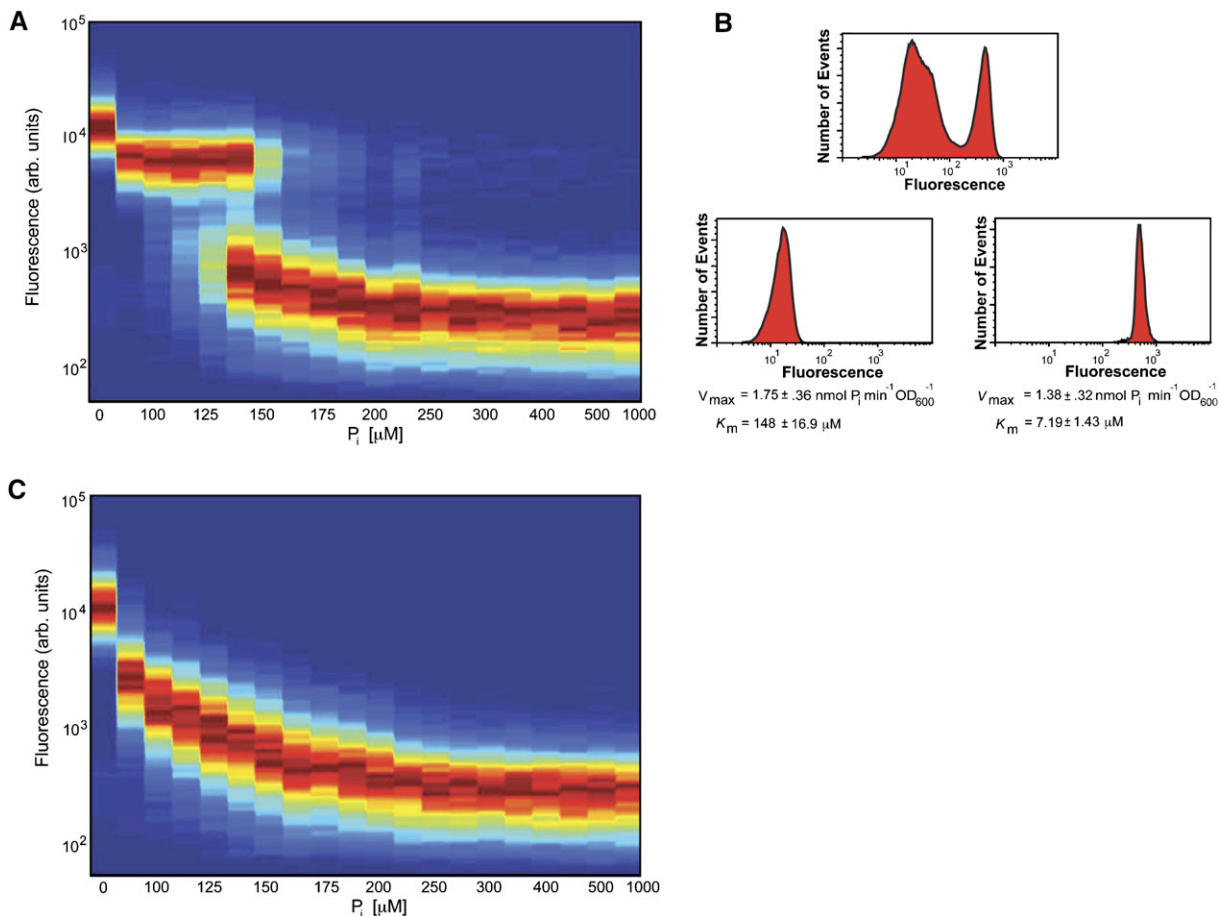


Figure 3. Positive Feedback through Induction of *Spl2* Generates Complex Population Dynamics

(A) Phosphate titration contour maps demonstrate the bistable response of wild-type cells in intermediate phosphate conditions. Strain EY1995 expressing *PHO84pr-GFP* was grown in no-phosphate medium supplemented with the indicated concentrations of inorganic phosphate. Cells were grown at low density (10^4 – 10^5 cells per ml) to prevent phosphate depletion. After 18 hr of growth (at which time the levels of GFP expression had reached steady state), cells were harvested and subjected to flow cytometry. Titration contour plots were generated for each phosphate concentration condition by normalizing to maximum peak height and creating a color map for all concentration measurements.

(B) Phosphate uptake assays were conducted on subpopulations of wild-type EY2095 (*PHO84pr-GFP*) cells grown in intermediate phosphate (150 μM phosphate) and sorted into populations expressing high and low levels of GFP by flow cytometry. The K_m values of the two populations suggest that low-affinity phosphate transporters dominate the uninduced population and high-affinity transporters predominate in the induced population.

(C) Strain EY2096 (*PHO84pr-GFP sp12Δ*) was grown and analyzed as in (A), and a contour map was generated from flow cytometry data.

downregulate the low-affinity transport system or, instead, keep the PHO pathway turned off and utilize low-affinity transporters for phosphate uptake. As the concentration of phosphate in the medium approaches the K_m for transport by the low-affinity transport system, phosphate uptake decreases and can no longer keep up with cellular usage, resulting in a drop in intracellular phosphate levels and activation of the PHO pathway. We speculate that the bimodality in *PHO84* expression arises under these conditions (intermediate phosphate) because cell-cell variability leads some cells to activate the PHO pathway and use high-affinity transporters, whereas others keep the pathway repressed and instead take up phosphate with low-affinity transporters. In intermediate phosphate conditions, these two states appear to be

equivalent solutions to obtaining phosphate, as their uptake rates are not distinguishable. The origin of this cell-cell variability in genetically identical cells is unclear—it may arise from noise in the signaling pathway (Maheshri and O’Shea, 2007) or from pre-existing differences between cells that bias them toward one fate or another. *sp12Δ* cells lacking the positive feedback loop cannot downregulate low-affinity transport, and when phosphate levels decrease to near the K_m of the low-affinity transport system, individual cells in the population respond similarly and produce enough Pho84 to allow them to balance phosphate uptake with usage. A graded response in *PHO84* results because, as phosphate levels drop further, more Pho84 is required to balance uptake with usage until eventually Pho84 is produced at maximal levels.

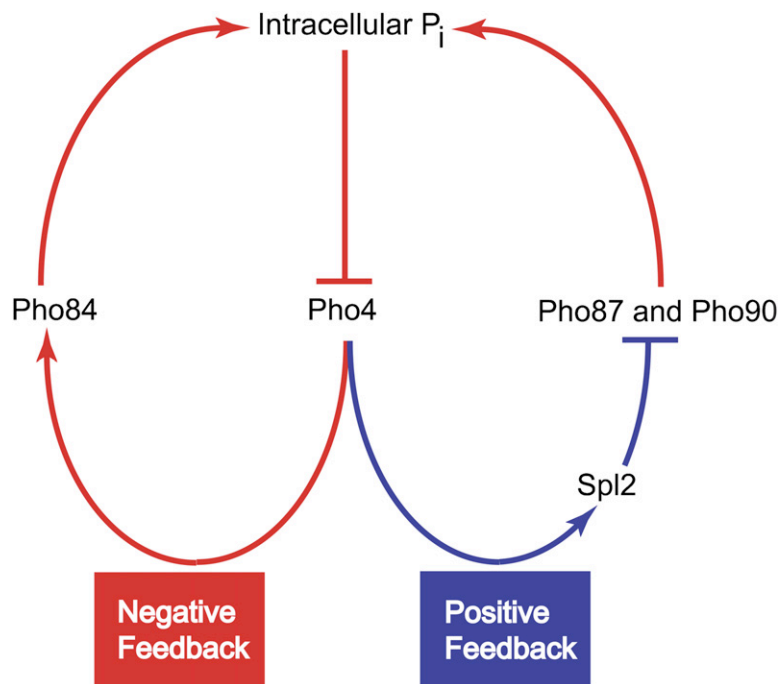


Figure 4. Phenotypic Switching of the Complement of Phosphate Transporters Is Mediated by Counteracting Positive and Negative Feedback Loops

Pho4, which is regulated by internal phosphate concentrations, controls the transcription of *PHO84* and *SPL2*. Pho84, because of its ability to transport inorganic phosphate, serves as a negative feedback element in the network. Spl2 is a positive feedback element because it downregulates the activity low-affinity phosphate transporters, reducing internal phosphate levels and leading to further activation of Pho4.

It is unclear why *S. cerevisiae* cells exhibit such a complex response to phosphate starvation—in particular, what benefit cells derive from generating two phenotypic states in intermediate phosphate and why low-affinity phosphate transport is downregulated in response to phosphate limitation. One rationalization is that the existence of two states may confer an advantage in an environment that includes conditions of phosphate limitation. It is also possible that the two states generated by feedback ensure that some fraction of the cells is optimally positioned to respond to a change in phosphate availability. For example, cells that already express Pho84 should respond faster to a change to phosphate starvation conditions than those utilizing low-affinity transporters. Therefore, feedback may allow the cells to carry out a form of “bet hedging” (Kussell and Leibler, 2005; Wolf et al., 2005). Yet another possibility is that transporter switching is merely a consequence of a system design utilized for a different property.

This work points to the difficulty in interpreting mutant phenotypes when genes are embedded in pathways containing feedback loops. The phenotypes of the *pho84Δ* strain have been difficult to rationalize, given that little Pho84 protein is present under the conditions where striking phenotypes are observed (Bun-Ya et al., 1991; Lau et al., 2000). With knowledge of feedback in the PHO pathway, these phenotypes can now be understood to arise from hysteresis in the *pho84Δ* strain. In the *pho84Δ* strain, which lacks polyphosphate stores (Ogawa et al., 2000), activation of the PHO pathway may result from phosphate demands that fluctuate during cell growth and division in high-phosphate conditions (Neef and Kladde, 2003).

Such fluctuations would lead to only transient activation of the PHO pathway in wild-type cells, but in *pho84Δ* cells, they likely trigger positive feedback through induction of *SPL2* and downregulation of low-affinity transport, which further reduces internal phosphate and drives the system to full activation of the PHO pathway. In contrast, wild-type cells have a polyphosphate buffer that is mobilized in times of phosphate limitation to replenish intracellular stores (Thomas and O’Shea, 2005), and even if PHO pathway activation occurs, wild-type cells balance *SPL2* induction with *PHO84* upregulation so that phosphate uptake is maintained. Therefore, the constitutive PHO pathway activation observed in the *pho84Δ* strain results from unconstrained positive feedback, not from a direct role for Pho84 in phosphate uptake in high-phosphate conditions.

SPL2 is a Pho4-regulated gene and was originally identified in a screen for genes that, when overexpressed, suppress the temperature sensitivity of a *plc1Δ* strain (Flick and Thorner, 1998). This study revealed that several perturbations and conditions that cause activation of the PHO pathway suppress the *plc1Δ* temperature sensitivity, suggesting that *SPL2* was isolated because it activates the PHO pathway and bypasses the Plc1 requirement at elevated temperatures. Spl2 may directly or indirectly downregulate low-affinity transporters; if it acts directly it may target the low-affinity phosphate transporters during their trafficking or at the plasma membrane.

It is striking that other nutrient signal transduction pathways, such as the galactose utilization (GAL) pathway in budding yeast and the lac operon in *E. coli* (Acar et al., 2005; Ozbudak et al., 2004), contain embedded positive

and negative feedback loops and respond in a similar switch-like manner. Additionally, nutrient-responsive signaling systems in budding yeast that share no sequence homology at the protein level have a similar pathway architecture consisting of regulated high-affinity transport and a low-affinity transport system (Bird et al., 2004; Harris et al., 2001; Jensen and Culotta, 2002; Tomas-Cobos et al., 2004; Waters and Eide, 2002). Feedback generated by this design may be an important feature of the ability to maintain nutrient homeostasis in the face of variable conditions.

EXPERIMENTAL PROCEDURES

Strain Construction

All yeast strains utilized in this study are listed in Table S1. Yeast media have been previously described (Wykoff and O'Shea, 2001). Methodologies for gene disruption have been detailed in the Supplemental Data.

Phosphate Uptake and Derivation of Kinetics

Uptake assays were performed (Wykoff and O'Shea, 2001) with final concentrations of phosphate assayed for initial velocity in a 3 min period being 5 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M, 150 μ M, 250 μ M, 500 μ M, 750 μ M, 1 mM, 2.5 mM, and 5 mM. For high-phosphate measurements, *pho84 Δ* strains were grown in midlog phase for 24 hr, minimizing the downregulation of phosphate uptake observed previously (Wykoff and O'Shea, 2001). Aggregate V_{\max} and K_m measurements were determined by reciprocal plots of at least eight data points. Reported means and standard deviations are from at least three independent experiments.

Assay for Growth on Plates

Cultures were grown to stationary phase overnight at 30°C in SD medium (or galactose containing medium when required), diluted to OD \sim 0.3, and serially diluted 10-fold. Approximately 3 μ l was spotted onto media containing agar with a pinning tool and allowed to grow at 30°C. Plates were photographed with a FlourChem 880 (Alpha Innotech, CA), and digital images were scaled with Adobe Photoshop 6.0 (Adobe Systems, CA) so that colony details could be readily visualized.

Flow Cytometry

Overnight cultures were diluted and grown to an OD₆₀₀ of 0.1. The culture was then diluted to 1×10^4 cells/ml into SD supplemented with inorganic phosphate concentrations ranging from 0 to 1 mM. Cultures were grown for 12–18 hr, diluting every 4 hr to ensure cells do not alter the phosphate concentration in the medium. Cells were sonicated to avoid aggregation and studied via flow cytometry with an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). Titration contour plots were generated for each phosphate concentration. To do so, semilog distributions of cell counts as a function of fluorescence intensity were 1D interpolated and normalized by the maximum cell count. These data were then concatenated, and steady-state fluorescence intensity was plotted as a function of phosphate concentration to create a FACS density series. All analysis was conducted with MATLAB (Mathworks, Natick, MA).

Cell Sorting

Strains were sorted with a cytometer MoFlow cell sorter (Dako, CA) in 10 mM NaCl and 150 μ M KH₂PO₄ solution and sorted into SD medium containing 150 μ M KH₂PO₄. Post-sorted cells were recovered in SD with 150 μ M phosphate medium for 5 hr and then concentrated 1000-fold and subjected to ³²P uptake (Wykoff and O'Shea, 2001) or immunoblot analysis. To ensure that *PHO84* expression was not

altered by the sorting and recovery, cells were reanalyzed by flow cytometry.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, five figures, and one table and can be found with this article online at <http://www.molecule.org/cgi/content/full/27/6/1005/DC1/>.

ACKNOWLEDGMENTS

The authors thank Terry Shroyer for his initial observations regarding suppression of the *pho84 Δ* phenotypes and the O'Shea laboratory, Narendra Maheshri, Naama Barkai, and Bodo Stern for helpful discussions. This work was supported by the National Institutes of Health (GM51377) and the Howard Hughes Medical Institute.

Received: April 27, 2007

Revised: June 10, 2007

Accepted: July 18, 2007

Published: September 20, 2007

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