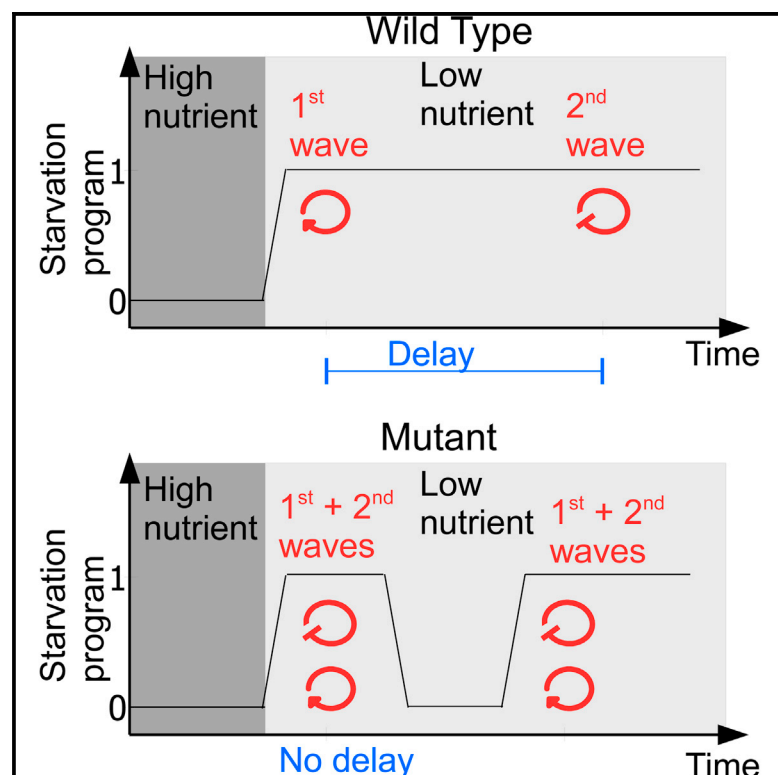


Sequential Feedback Induction Stabilizes the Phosphate Starvation Response in Budding Yeast

Graphical Abstract



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In Brief

Cells depleted of nutrients activate processes that increase intracellular nutrients, which could destabilize the overall response. Vardi et al. show that, in budding yeast, the stability of the phosphate starvation response depends on a precise temporal pattern: an initial induction of a positive feedback preceding the induction of major correction mechanisms.

Highlights

Cells induce the phosphate starvation response genes in two waves

The first wave acts through positive feedback to stabilize the response

The second wave acts as destabilizing negative feedback

Preventing the delay between the waves leads to transient shut-down of the response

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Sequential Feedback Induction Stabilizes the Phosphate Starvation Response in Budding Yeast

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SUMMARY

Depletion of essential nutrients triggers regulatory programs that prolong cell growth and survival. Starvation-induced processes increase nutrient transport, mobilize nutrient storage, and recycle nutrients between cellular components. This leads to an effective increase in intracellular nutrients, which may act as a negative feedback that downregulates the starvation program. To examine how cells overcome this potential instability, we followed the transcription response of budding yeast transferred to medium lacking phosphate. Genes were induced in two temporal waves. The first wave was stably maintained and persisted even upon phosphate replenishment, indicating a positive feedback loop. This commitment was abolished after 2 hr with the induction of the second expression wave, coinciding with the reduction in cell growth rate. We show that the overall temporal stability of the expression response depends on the sequential pattern of gene induction. Our results emphasize the key role of gene expression dynamics in optimizing cellular adaptation.

INTRODUCTION

Cells depend on their external environment for supplying essential nutrients. When the levels of nutrients fluctuate, regulatory mechanisms adjust protein level and function to optimize growth and survival. Induced mechanisms include optimization of nutrient transport, mobilization of nutrient into or out of storage, scavenging extracellular resources for nutrients, and recycling nutrients among cellular components. In addition to mechanisms specifically tailored for the particular starvation, several global processes are also regulated upon nutrient depletion, including the induction of general stress genes, repression of ribosomal associated genes, and modulation of the rate of biomass production and cell division rate (Brauer et al., 2008).

Genes involved in the starvation program have been characterized in many systems, yet less attention has been given to the dynamics by which this response is induced. This dynamics is subject to multiple constraints; on one hand, rapid response is beneficial as it enables correcting potential limitations before cellular functions are disturbed. On the other hand, activating the starvation program too early may reduce cellular fitness, because this early induction may deplete resources that would otherwise be allocated to alternative cellular functions. It may be further beneficial to dynamically adjust the activated processes based on the level of limitation: a response that is too mild may not be sufficient to overcome the limitation, whereas an adaptation mechanism that increases the intracellular nutrient too rapidly may trigger a negative feedback that downregulates its own induction. How these constraints impact the temporal response to nutrient limitation is not well understood.

Budding yeast respond to a reduction in intracellular phosphate by rapidly translocating the transcription factor Pho4 to the nucleus (Kaffman et al., 1994, 1998). Following its activation, Pho4 directly induces the expression of target genes denoted collectively as the Pho regulon. As part of this induction, high-affinity phosphate transporters are strongly expressed, whereas activity of the low-affinity transporters is downregulated (Bun-Ya et al., 1991; Wykoff et al., 2007). We recently reported that when external phosphate is gradually depleted, this dual transporter system prolongs the lag time from Pho4 activation until the onset of growth limitation (Levy et al., 2011). This is because activation of Pho4 occurs when external phosphate is depleted to about the dissociation constant of the low-affinity transporters, whereas growth limitation occurs when phosphate level is reduced further, to concentrations comparable to the dissociation constant of the high-affinity transporters. When the lag time between Pho4 activation and the onset of the growth limitation (so-called preparation time) is impaired, cells delay their recovery from phosphate starvation (Levy et al., 2011).

When external phosphate is abruptly depleted from the environment, induction of the high-affinity transporters, by itself, does not contribute to elevating intracellular phosphate. Growth could still be prolonged, however, by other mechanisms. For example, cells secrete phosphatases that retrieve phosphate from substrates available in the extracellular environment

(Lenburg and O'Shea, 1996; Ogawa et al., 2000; Shnyreva et al., 1996). Other induced genes retrieve phosphate from internal sources, by mobilizing dedicated storage such as vacuolar PolyP (Ogawa et al., 2000), or by dephosphorylating intracellular substrates such as nucleotides or peptides (Donella-Deana et al., 1993; Reddy et al., 2008; Xu et al., 2013). Indeed, cells transferred to medium lacking phosphate continue to divide for a couple of generations before arresting their division cycle.

Because Pho4 activation depends on the depletion of intracellular phosphate, efficient action of the Pho regulon genes in increasing intracellular phosphate could function as a negative feedback loop to downregulate Pho4 activity, thereby inhibiting their own expression. However, such instability has not been reported. To better understand how cells coordinate their response to phosphate depletion and how they ensure the stability of this response, we followed the transcription profile of budding yeast transferred to medium that lacks phosphate at high temporal resolution. We found that Pho4-dependent genes are induced in two temporal waves. The first wave introduces a stabilizing positive feedback loop, whereas the second (which includes the majority of extracellular and intracellular phosphatases) acts as an effective negative feedback. Notably, the second induction wave coincides with the reduction in growth rate and upregulation of the environmental stress response. The destabilization effect of the second induction wave depends on specific genes, which we identify. Furthermore, the temporal stability of the expression program is abolished when eliminating the delay in the second expression wave induction. We discuss the implications of our results for better understanding the means by which cells adapt to phosphate starvation.

RESULTS

Genome-wide Transcription Profiles Show a Temporal Response to Phosphate Depletion

We characterized the genome-wide transcription profile induced by phosphate starvation using high-throughput RNA sequencing. Log-phase budding yeast cells were transferred from a rich medium to a medium lacking phosphate and were followed for 6 hr, with measurements taken every 15 min (Figure 1A). To focus on the changes in gene expression, we report the (log) expression ratio relative to cells growing in rich medium (Figure 1B, left).

The overall similarity between the profiles at different time points can be quantified using the (Pearson) correlation. Samples taken during the first 2 hr were well correlated ($\langle c \rangle = 0.69$), and an even higher correlation was observed between samples from the later time points ($\langle c \rangle = 0.87$). In contrast, correlation between early and late samples was significantly lower ($\langle c \rangle = 0.59$) (Figure 1B, right).

A possible interpretation of this correlation pattern is that the response is biphasic, with an initial change in gene expression triggered immediately upon transfer to medium lacking phosphate, followed by a second wave of expression change occurring about 2 hr later. Two Pho4-dependent genes, *PHO84* and *PHO5*, have previously been reported to be induced with different temporal kinetics upon transfer to phosphate-lacking medium: *PHO84* is induced immediately upon transfer, whereas

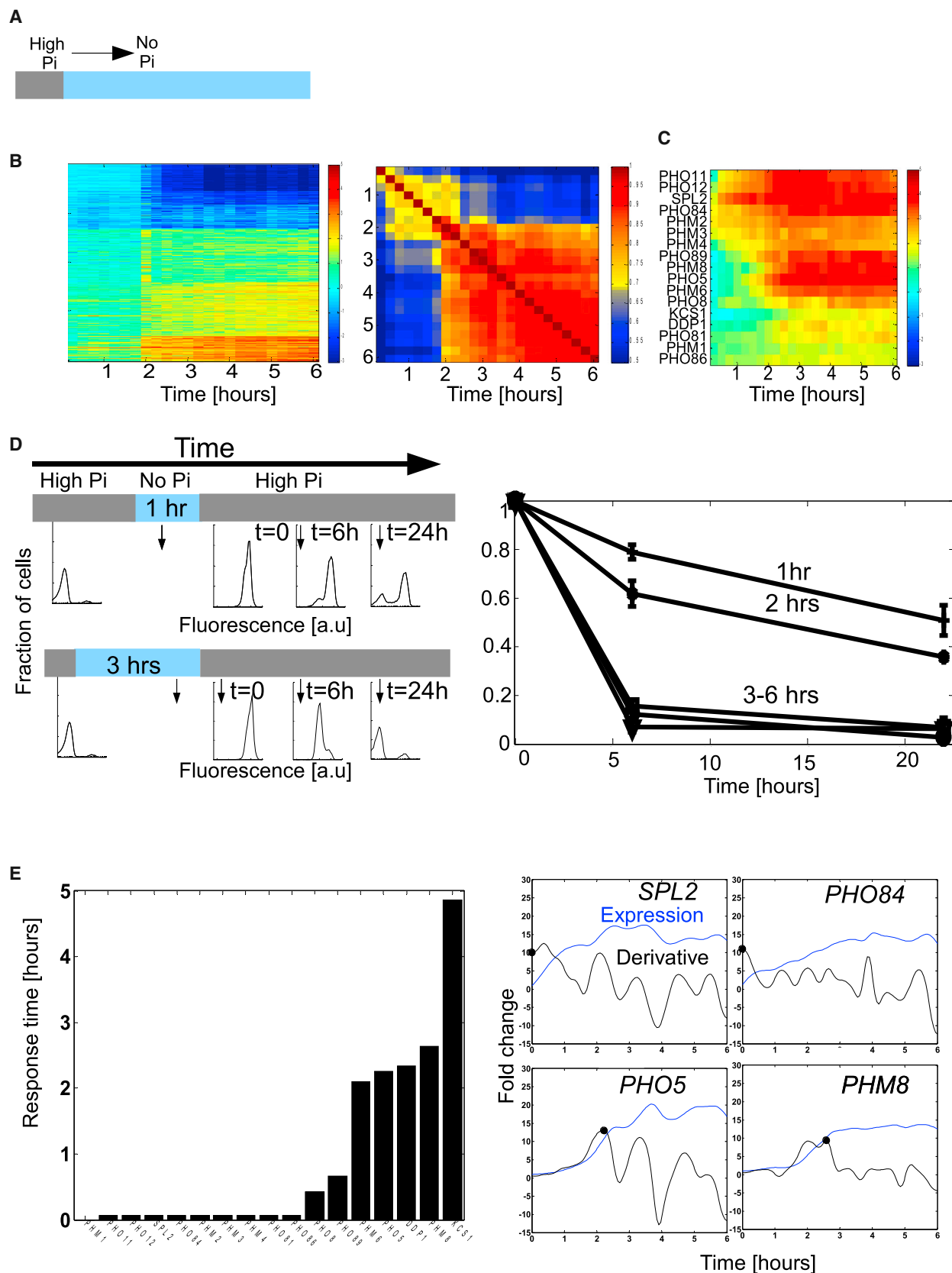
PHO5 induction follows with a delay (Thomas and O'Shea, 2005). Our data similarly showed that *PHO84* was induced immediately upon phosphate depletion, whereas *PHO5* was induced later, about 2 hr following the transfer to phosphate-lacking media (Figures 1C and 1E).

We focused on 17 Pho regulon genes that have been shown to be induced by limited phosphate and have a well-defined role in phosphate homeostasis. The induction time of each gene was defined by the first time point showing a maximal rate of expression increase (derivative). Induction times were bimodal: about half of the genes were induced immediately upon the transfer, showing maximal induction rate already at the first time point examined (Figures 1C and 1E). The other genes were induced with a delay, which was roughly 2 hr in most cases. Notably, most of the rapidly induced genes showed a second induction peak that was again localized around the 2 hr time point (Figures 1C and 1E). Therefore, the Pho regulon is induced in a well-defined temporal order: some genes are induced rapidly upon phosphate depletion, whereas other genes are induced with a significant delay of about 2 hr.

Rapidly Induced Genes Stabilize Pho4 Activity

We asked whether the temporal order by which the Pho genes were induced reflects functional differences between the early and late genes. Among the rapidly induced genes were both genes whose function increases internal phosphate levels, as well as genes that could reduce the level of internal phosphate. Genes that function to increase the level of internal phosphate include the high-affinity transporter *PHO84*, its auxiliary factor *PHO86* (Lau et al., 2000), and the two secreted acid phosphatases *PHO12* and *PHO11*, which retrieve phosphate from external sources (Shnyreva et al., 1996). Genes that could act to reduce the level of internal phosphate include the four *PHM1-4* genes, which direct internal phosphate into vacuolar PolyP storage (Ogawa et al., 2000) and *SPL2*, which downregulates the activity of low-affinity transporters (Wykoff et al., 2007). *PHO81*, which also belongs to this group, promotes Pho4 activity by inhibiting the inhibitory kinase Pho85-Pho80 (O'Neill et al., 1996; Schneider et al., 1994).

Therefore, based on their function, the rapidly induced genes could potentially introduce either a negative or positive feedback loop acting on Pho4 activity. Although neither of these feedbacks is necessarily effective, our previous study suggests that the induced genes initiate a positive feedback (Vardi et al., 2013), because we found that cells growing at steady state in intermediate phosphate levels activate a positive feedback loop that depends on the induction of the *SPL2* and *PHM3-4* genes. We therefore hypothesized that, also in the present, dynamic context, the first induction wave introduces an effective positive feedback loop. To examine this possibility, we replenished phosphate to the starved cells and examined their recovery. In the absence of a positive feedback loop, we expect an immediate downregulation of all induced genes. In contrast, if cells activate a positive feedback loop that stabilizes Pho4 activity, cells will maintain the Pho4-dependent genes induced even if phosphate is replenished and will downregulate them only upon some stochastic event that may break the positive feedback loop (Vardi et al., 2013).



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Cells were transferred to a medium lacking phosphate for varying amounts of time and were then shifted back to rich media (Figure 1D). A yellow fluorescence reporter driven by the *PHO84* promoter *PHO84pr-YFP* was used to follow the expression of a rapidly induced Pho4-dependent promoter at the single-cell level. The single-cell distribution of this reporter expression was quantified using flow cytometry throughout the time course. High reporter expression was observed in all cells following 1 hr in no-phosphate media (Figure 1D). Notably, a large fraction of the cells that were subject to phosphate starvation for 2 hr or less maintained reporter expression for over 20 hr following transfer back to rich medium, while undergoing at least 12 divisions (Figure 1D). Single cells downregulated reporter expression at stochastic times, consistent with noise-driven destabilization of the positive feedback loop (Vardi et al., 2013). This ability to maintain reporter expression in the presence of high phosphate was lost in cells deleted of the rapidly induced genes *SPL2* and *PHM3* (Figure S1). We conclude that the first wave of gene induction leads to a stabilizing positive feedback that maintains Pho4-dependent gene expression even when phosphate is replenished.

The Second Wave of Pho Gene Expression Acts as a Destabilizing Negative Feedback

In contrast to cells grown at intermediate levels of phosphate, which maintain a stable induction of Pho4-dependent genes when phosphate is replenished, steady-state cultures growing at low phosphate downregulate these genes immediately upon phosphate replenishment (Vardi et al., 2013). Consistent with this finding, cells treated with medium lacking phosphate for 3 hr or longer downregulate *PHO84pr-YFP* reporter expression immediately upon their return to rich media (Figure 1D). This loss of stability could result from the downregulation of the positive feedback associated with the rapidly induced genes. These genes, however, remained highly expressed throughout the time course, and their expression in fact increased, rather than decreased, at the 2 hr time point (Figure 1C). Alternatively, the second wave of Pho4-dependent genes induced at about the 2 hr time point could introduce an effective negative feedback that dominates over the earlier induced positive feedback.

The possibility that the delayed genes introduce an effective destabilizing feedback is supported by their functional association.

This group includes phosphatases that are either secreted (*PHO5*) or act on cellular substrates (*PHM8*, *PHO8*, *DDP1*). Also in this group, we find *PHO89*, a high-affinity phosphate transporter and *PHM6*, a protein of unknown function (Figure 1E). Furthermore, *KCS1*, an additional gene that belongs to this group, functions to reduce IP7, a major signaling molecule implicated in Pho4 activation in response to phosphate depletion (Lee et al., 2007). Therefore, all these genes are expected to decrease Pho4 activation either directly or by increasing intracellular phosphate and may introduce an effective negative feedback (Figure 2A).

We reasoned that, if genes induced during the second temporal wave destabilize the starvation response, their deletion will maintain the stabilizing effect of the initial induction wave for a longer time. To test this, we examined cells deleted of four of these genes, *PHO5*, *PHM8*, *PHM6*, and *PHO8*, individually or in combination, and tested whether they maintain expression of Pho4-dependent genes when phosphate is replenished. Indeed, deleting both *PHM8* and *PHM6* ($\Delta phm6\Delta phm8$) prolonged the time in which the induction of Pho4-dependent remained stable (Figure 2B). We also examined whether constitutive expression of the second wave genes destabilizes the response by engineering cells to constitutively express the internal phosphatase *PHO8*, driven by the *TDH3* promoter. Indeed, constitutive expression of *PHO8* significantly reduced the fraction of phosphate-starved cells, which maintained the Pho4-dependent gene expression upon phosphate replenishment (Figure 2B). We conclude that the second wave of Pho4-dependent gene induction introduces an effective negative feedback loop that overcomes the preceding positive feedback loop and can destabilize Pho4 activation, at least when cells are transferred from phosphate starvation conditions to rich media.

Preventing the Temporal Delay in Pho4-Dependent Gene Expression Results in a Transient Oscillatory Behavior

Wild-type cells growing in medium lacking phosphate maintain a stable induction of Pho4-dependent genes, indicating that cells overcome the inherent negative feedback associated with phosphatases expression. We asked whether the delay between the first, stabilizing wave, and the second, destabilizing wave, is required for this stable expression.

Figure 1. Biphasic Transcription Response to Phosphate Depletion

- (A) Time resolved transcription profiling. Experimental design: log-phase cells were transferred to no-phosphate medium and were followed for 6 hr, with samples taken every 15 min. The transcription profile at each time point was defined using high-throughput sequencing.
- (B) Biphasic modulation of gene expression. Expression values were normalized by their levels prior to transfer, to capture changes in expression. Shown is the (Log2) expression of genes that were induced or repressed more than 3-fold during the time course (left). Similarity between samples was defined based on the (Pearson) correlation between the (log2) expression changes and is shown here for the 24 time points profiled (right). Note the high similarity between samples taken during the first 2 hr and the high similarity between samples taken at the later time points.
- (C) Temporal induction of Pho genes. A set of 17 genes involved in phosphate homeostasis and induced by low phosphate were assembled based on literature. Shown is the (log2) expression of each of those genes relative to its expression at high phosphate.
- (D) First expression wave coincides with commitment to Pho4 activation. The stability of Pho4 activation was tested using the *PHO84pr-YFP* reporter whose expression is induced at low-Pi in a Pho4-dependent manner. Cells were transferred to a medium lacking phosphate for the indicated amount of time before shifted back to rich medium. The distribution of single-cell reporter expression was quantified using flow cytometer. Shown is the fraction of cells that maintained reporter expression as a function of time in rich medium. Note that a large fraction of cells exposed to no-Pi media for 2 hr or less maintained reporter expression in rich medium (commitment), whereas longer exposure was followed by an immediate downregulation of reporter expression. Error bars stand for SD.
- (E) Immediate versus delayed induction of Pho genes. The time of gene induction was defined as the first time point in which the rate of expression change was maximal. Induction times are shown for the 17 Pho genes (left). Note that ten early genes are induced immediately upon the transfer, whereas seven others are induced at a delay that varies between 30 min to 2 hr. Plots of genes expression and its rate of change are shown for four representative genes, as indicated (right).

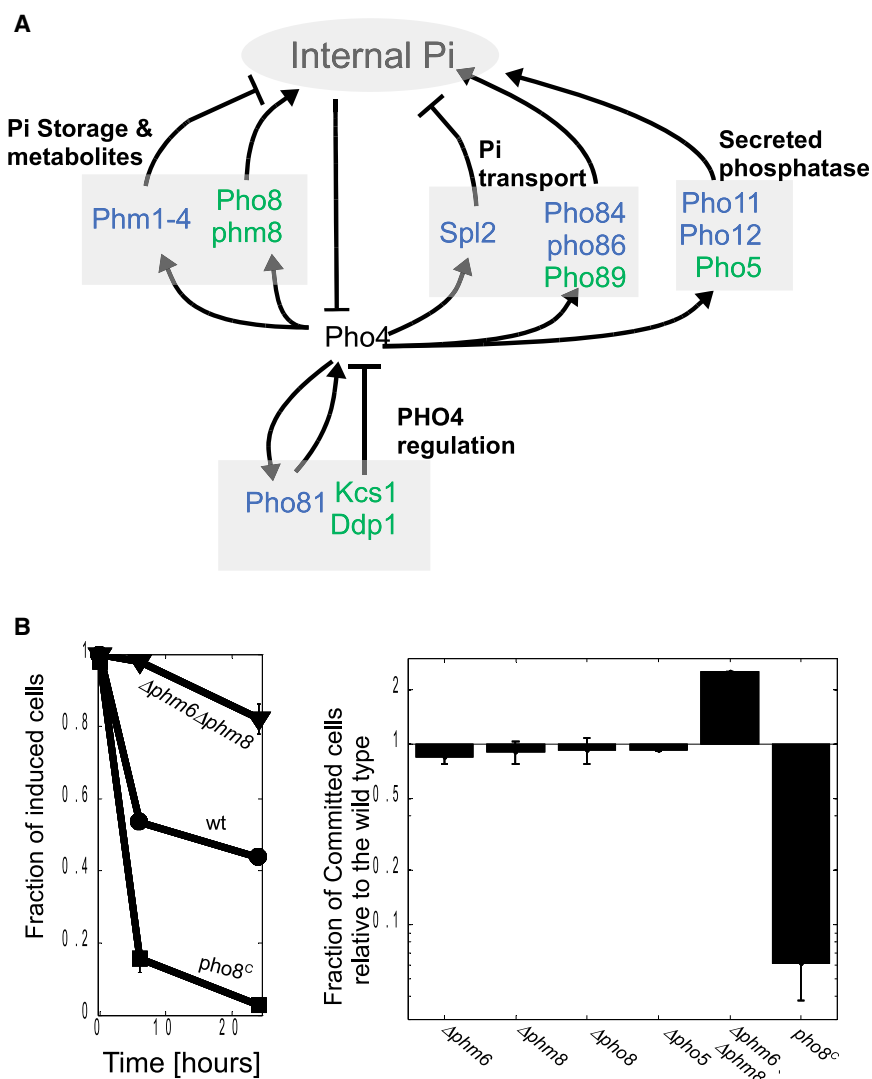


Figure 2. Delayed Induced Internal Phosphatases Destabilize Pho4 Activation

(A) Putative positive and negative feedbacks associated with induced Pho genes. A scheme summarizing the effect of different Pho genes on the activation of the Pho4 transcription factor. Pho4 is activated by depletion of intracellular phosphate. Different Pho genes function to increase or decrease internal phosphate levels, as indicated. Additional, more direct feedbacks are defined by pathways transmitting reduced phosphate level to Pho4 activation. Rapidly induced genes are marked in blue, whereas those induced with a delay are marked in green.

(B) Commitment to Pho4 activation depends on late-induced genes. Commitment was assayed as described in Figure 1D for strains of the indicated background. Note the reduction in committed cells in a strain deleted of the two delayed genes *PHM6* and *PHM8* and the increased fraction of committed cells in a strain that constitutively expressed the delayed gene *PHO8* using the *TDH3* promoter (*pho8^c*). Error bars stand for SD.

each strain showed a different oscillation pattern: cells in which Pho85 expression was reduced underwent two oscillations, whereas the other strains showed a single oscillation. We conclude that the delayed expression of Pho genes is required for maintaining their stable induction.

The Delayed Wave of Pho4-Dependent Expression Coincides with ESR Induction and a Reduction in Growth Rate

Activation of Pho4 depends on the level of intracellular phosphate (Auesukaree et al., 2004). The two induction waves

To examine this, we considered mutants in which all Pho genes are rapidly induced. Cells deleted of *PHM3* or *PHM4* are devoid of stored PolyP and were previously shown to induce *PHO5* immediately upon phosphate depletion (Thomas and O'Shea, 2005). We expected a similar rapid induction of all Pho4-dependent genes in both mutants. We further expected a stronger and faster activation of Pho4 in cells showing a 2-fold reduction in the expression of *PHM3* or of *PHO85*, a negative regulator of Pho4 (Vardi et al., 2013). Indeed, repeating our time-resolved transcription profiling with these four mutants verified that the full Pho regulon was simultaneously induced immediately upon transfer to medium lacking phosphate (Figure 3, left).

Notably, in contrast to the stable induction of this transcription program in wild-type cells, the rapid induction of the Pho genes in these mutants was followed by their pronounced downregulation. This downregulation was transient and was followed by a subsequent induction (Figure 3). The timing and extent of the downregulation varied between strains. The qualitative transient oscillatory behavior was observed in all four cases, although

may therefore reflect two activation thresholds, whereby the lower threshold is defined by a sharper decrease in intracellular phosphate or by the depletion of PolyP storage (Thomas and O'Shea, 2005). We asked whether these thresholds can also explain the more general stress responses induced by phosphate starvation.

In the budding yeast, a variety of environmental stresses trigger a common gene expression program, termed the Environmental Stress Response (ESR) (Causton et al., 2001; Gasch et al., 2000). In our data as well, stress genes were upregulated, whereas genes associated with the making of ribosomes were downregulated (Figure 4A, top). The ESR is typically induced rapidly, within minutes of exposure to stress (Gasch et al., 2000). In sharp contrast, this program was only slightly induced immediately upon phosphate depletion, but was strongly activated at about 2 hr following transfer to medium lacking phosphate, coinciding with the second wave of Pho gene induction (Figure 4A, top). This delayed response was even more pronounced when examining expression of genes involved in

ribosomal biogenesis (the Ribi module); here, no reduction in expression was observed until 2 hr following the transfer to phosphate depleted medium. Rather, this group underwent a strong and uniform decrease in expression at the 2 hr time point (Figure 4A, top).

The induction of stress genes and downregulation of Ribi genes remained correlated with the expression of the second wave of Pho gene in all of the mutants showing rapid and synchronized induction of the full Pho regulon as well. Thus, in the *PHM3/4* mutants and in cells with reduced *PHO85* expression, the ESR was induced rapidly upon transfer to medium lacking phosphate. Furthermore, ESR activation showed the same transient oscillatory behavior observed for the Pho genes (Figure 4A). Because ESR activation does not depend on the Pho4 transcription factor, this coordinated oscillatory behavior supports the notion that the ESR is triggered by the sharp decrease in internal phosphate, and that the negative feedback loop introduced by the second wave genes acts by effectively increasing internal phosphate.

Changes in the ESR gene expression program often correlate with changes in cell growth rate (Brauer et al., 2008). We asked whether growth rate changes at the time of ESR induction. Cells transferred to a medium lacking phosphate underwent on average two additional divisions before arresting their cell cycle. Notably, average population growth was rapid during the first 2 hr and decreased only at about the 2 hr time point following transfer, correlating with the second wave of Pho gene induction and ESR activation (Figure 4B).

We also measured the growth rate of cells deleted of *PHM3*, in which the decrease in internal phosphate is expected to be faster due to the lack of PolyP storage. Growth rate was largely reduced compared to wild-type cells, although cells still underwent almost two cell divisions before arresting. Here as well, a sharp reduction in growth rate was observed upon the transfer to a media lacking phosphate, consistent with the immediate induction of the ESR program (Figures 4A and 4B).

Pho4-Dependent Gene Induction Precedes ESR Activation and Growth Rate Reduction in Mutants, Showing Delayed Pho4 Activation

We therefore concluded that, in wild-type cells and in cells that rapidly activate the full Pho regulon, the induction of the second wave Pho gene coincides with activation of the general stress response and a reduction in growth rate. To examine the generality of this temporal correlation, we analyzed the transcription program of mutants showing a delayed induction of the Pho regulon.

We first considered mutants deleted of *VIP1*, a gene required for the production of inositol heptakisphosphate (IP7) (Lee et al., 2007). Previous studies argued that IP7 is essential for Pho4 activation in low phosphate conditions and reported that cells deleted of *VIP1* do not induce Pho regulon genes within 2 hr of phosphate depletion (Lee et al., 2007, 2008). We verified this observation, but noted that the Pho regulon induction is not absent yet only delayed (Figure 5A, second row). The delay was most pronounced for the first induction wave, which was now observed only about 2 hr following transfer to phosphate-lacking media. The second induction wave was delayed to a

lesser extent and the induction of most of its genes was now observed less than half an hour after the first wave (Figure 5C). As expected from the significantly shorter time period between the two induction waves, commitment was lost in those cells (Figure S2).

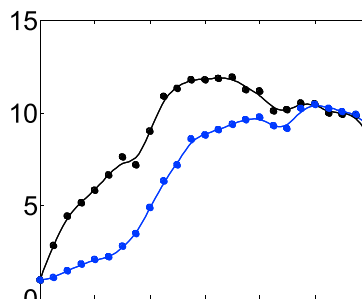
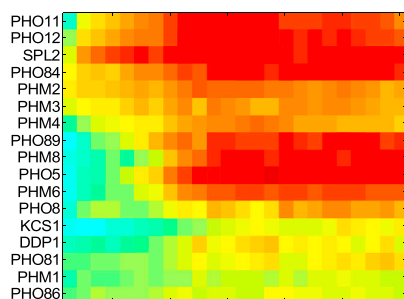
ESR induction was also significantly delayed in this mutant. Notably, the induction now occurred at about 1 hr following the second Pho induction wave (Figure 5A). Quantifying the growth rate of this mutant, we found that the phase of rapid growth rate was also prolonged, with a decrease in growth rate observed simultaneously with ESR activation (Figure 5D). Thus, the correlation between reduced growth rate and induction of the ESR was maintained, but both were now delayed relative to the second wave of Pho gene induction.

As a complementary approach for delaying Pho gene induction, we analyzed cells that overexpress the low-affinity transporter *PHO90*. We reasoned that these cells will delay Pho4 activation, as they likely accumulate additional PolyP (Hürlimann et al., 2009). This was indeed the case. Notably, also here, the delay in induction was most pronounced for the first wave of Pho genes and ESR genes, whereas the second wave of Pho genes was delayed to a lesser extent. As a result, Pho induction now preceded ESR activation and the reduction in growth rate (Figure 5B).

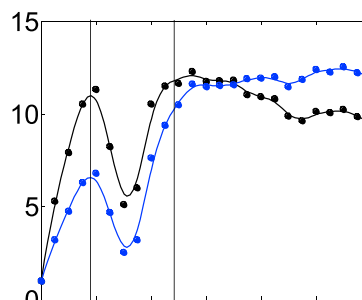
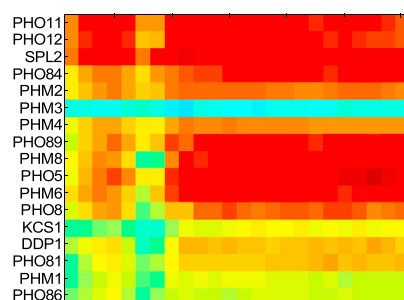
The Two Expression Waves Contribute Differently to the Phenotypic Cost of Pho Gene Expression

Delaying the induction of Pho4-dependent genes therefore breaks the temporal correlation between the ESR and the second Pho wave. The finding that the second wave of Pho expression preceded the reduction in growth rate suggested to us that their induction may reduce growth rate to a larger extent than expression of genes induced in the first wave. This possibility is further supported by the functional association of the genes induced in the second wave, some of which are internal phosphatases acting on cellular components such as nucleotides or peptides (Donella-Deana et al., 1993; Reddy et al., 2008; Xu et al., 2013). This functional association is in contrast to the first Pho wave genes, which do not appear to interfere with internal cellular processes.

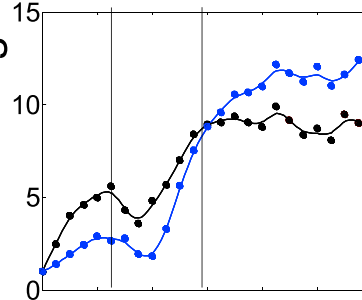
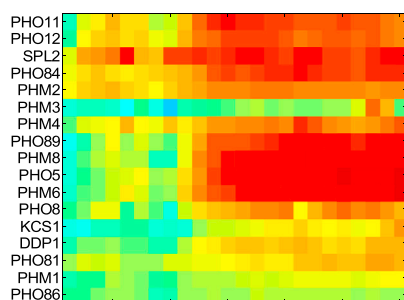
To define the extent by which induction of Pho genes interferes with other cellular processes, we forced their expression in rich media and used a sensitive competition assay to define the associated reduction in growth fitness. For the first Pho wave, we capitalized on the fact that its expression is maintained for many generations when cells are transferred back to rich media (Figure 1D). We therefore performed a direct competition between cells subject to 2 hr phosphate starvation prior to their return to rich media, and cells not subject to this treatment. Notably, we could not detect growth rate differences between the induced and uninduced cells (Figures 6A–6C). As a complementary approach, we considered a strain in which the wild-type Pho4 allele was replaced by its constitutively partially activated allele (Springer et al., 2003) (Figures 6B and 6C). This partially activated strain expresses most of the first-wave genes also in rich media (Springer et al., 2003). Here as well, we could not detect growth rate differences between wild-type and partially activated cells growing in rich media. We conclude that within



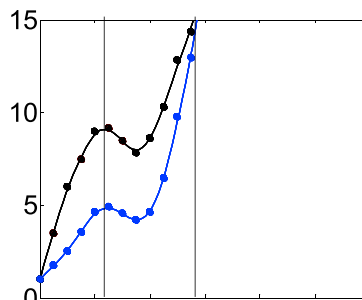
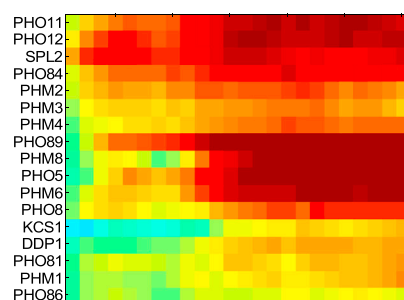
Wild type



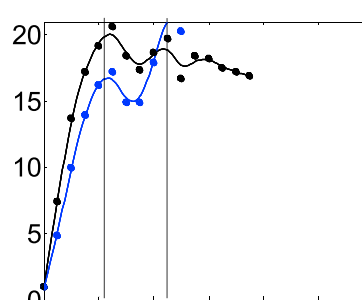
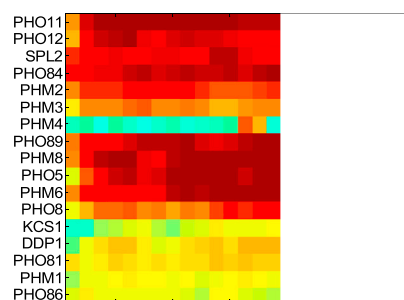
$\Delta phm3$



phm3
damp



Pho85
damp



$\Delta phm4$

Time [hours]

Time [hours]

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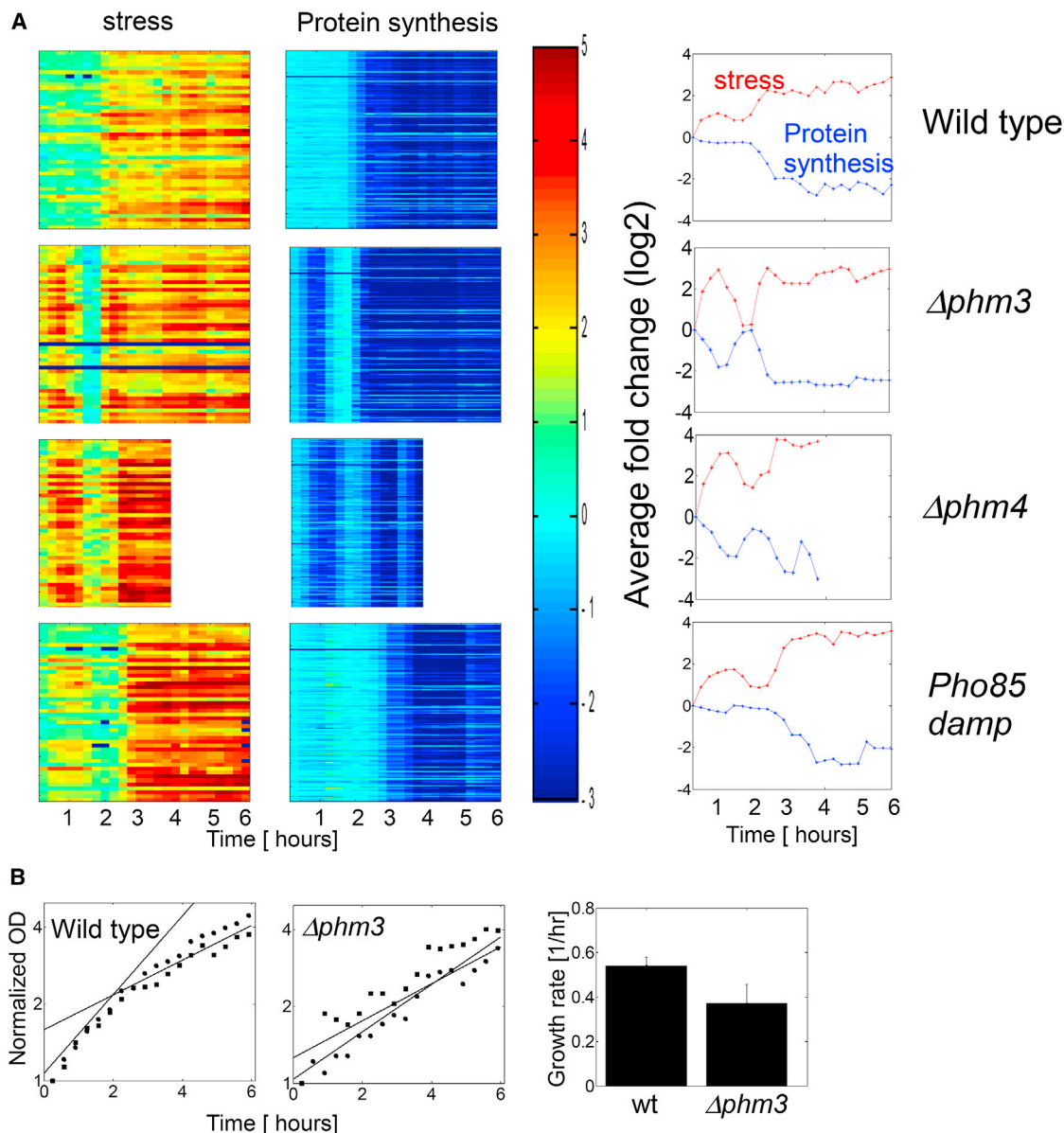


Figure 4. Delayed Activation of the Stress Response

(A) Stress-associated genes correlate with Pho regulon induction. Same as Figure 3 but for genes induced by general environmental stress (right, red curve) and genes associated with ribosomal biogenesis (middle, blue curve).

(B) Delayed reduction in growth rate. Growth rate of cells transferred to no-phosphate medium was quantified based on the optical density (OD), as shown. Note the decrease in growth rate 2 hr following the transfer, coinciding with the induction of the stress response. Error bars stand for SD.

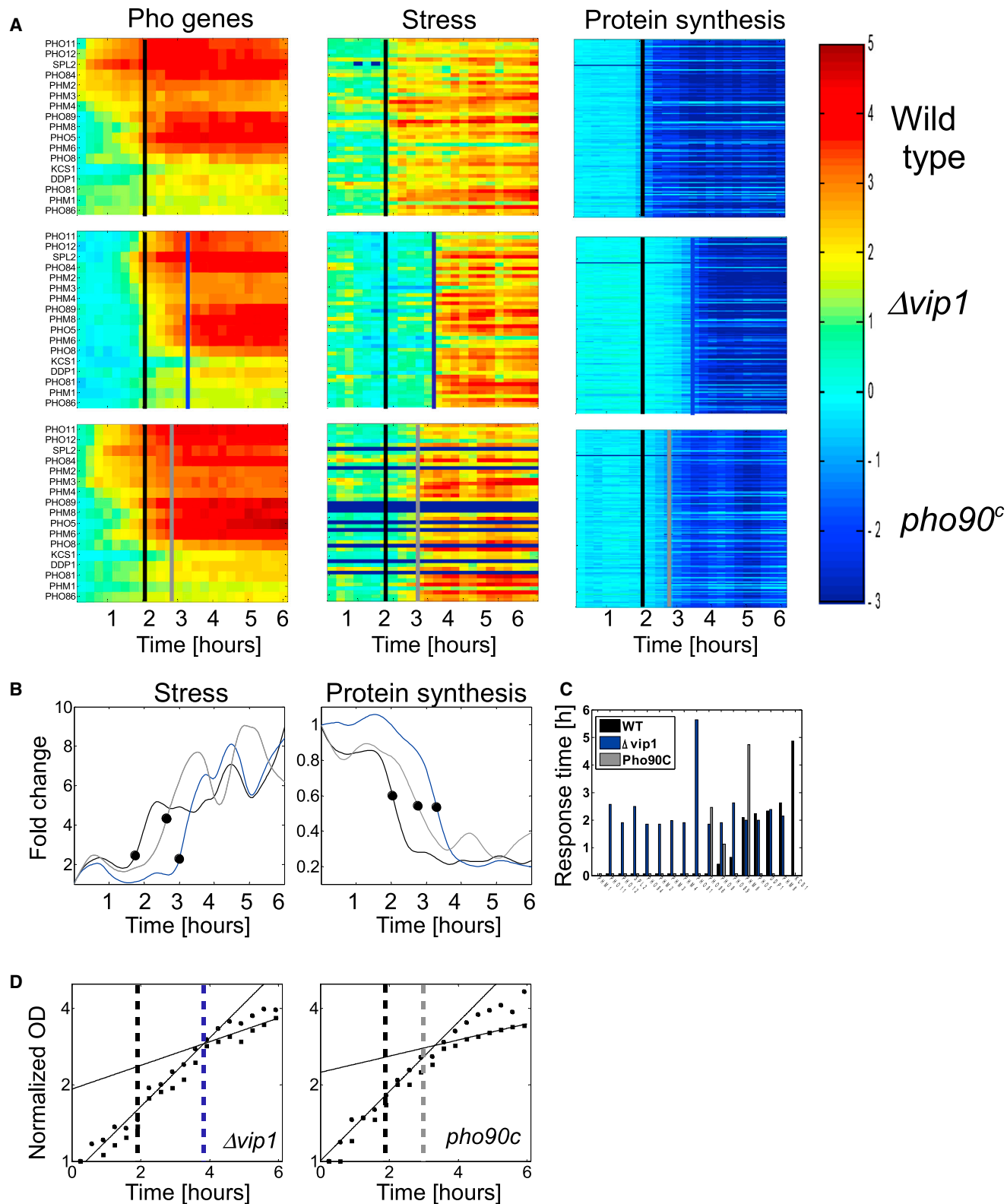
the sensitivity of our competition assay ($\sim 3\%$), expressing the first wave of Pho4-dependent genes does not reduce cell growth in rich medium.

We next tested the fitness cost of fully activating the Pho regulon. To this end, we examined cells deleted of *PHO80*, a cyclin

that together with *PHO85* inhibits Pho4. In *PHO80*-deleted cells, Pho4 is fully active regardless of external phosphate levels, meaning that it will be fully active in rich media. Cells deleted of *PHO80* showed a significant reduction in growth rate ($\sim 15\%$). Notably, this growth defect was fully rescued by Pho4

Figure 3. Early Induction of Delayed Pho Genes Results in Transient Downregulation of Pho Gene Expression

The time-resolved transcription profiling described in Figure 1 above was repeated for the indicated strains. Expression of individual Pho genes is shown on the left, and the average change in expression of the seven immediate (blue) or seven delayed (black) genes on the right (see the Experimental Procedures). Vertical lines indicate the maximal increase and decrease in expression change (see the Experimental Procedures).



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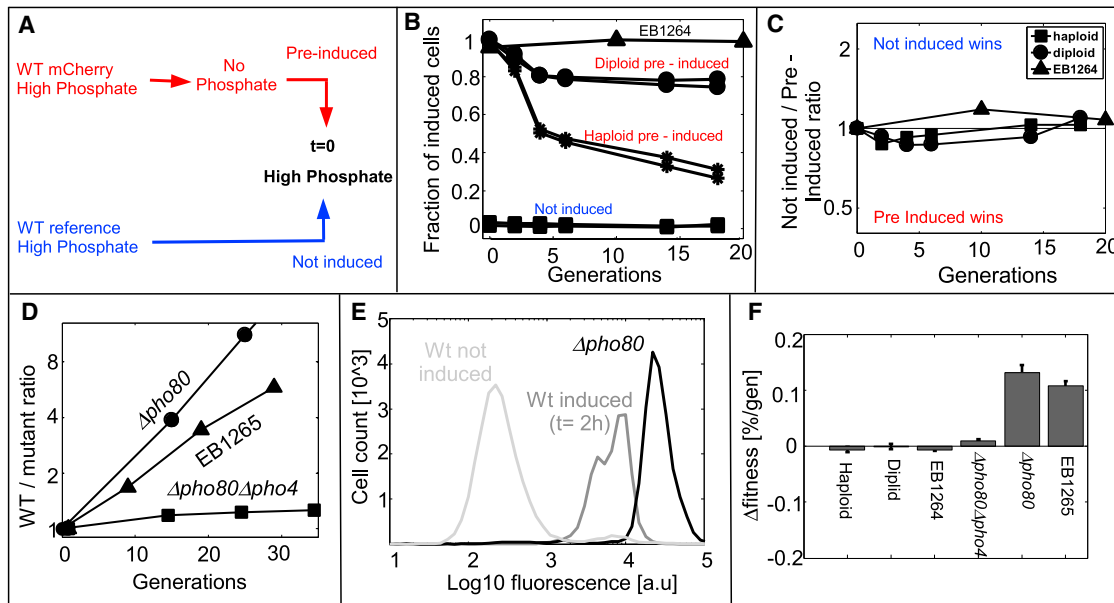


Figure 6. Fitness Cost of Activating the Phosphate Starvation Response in Rich Medium

(A–C) Partial induction of Pho genes does not reduce competitive growth in rich media. Committed cells were obtained by preincubation in medium lacking phosphate for 2 hr (A). Those cells maintained *PHO84pr*-YFP reporter induced for over 15 (diploids) or ten generations (haploids, B). In the EB1264 cells, *PHO4* was replaced by a partially active allele (Springer et al., 2003). Competition experiments were performed as described in Experimental Procedures. (D and E) Full induction of Pho genes reduces competitive growth in rich media. Competitive experiments were performed as described for cells of the indicated phenotype. Induction level of *PHO84pr*-YFP is measured by flow cytometry (E). (F) Fitness cost. Fitness cost of the different strains indicated is quantified based on the curves shown in (C) and (E). Error bars stand for SD.

deletion, confirming its origin in Pho gene expression (Figures 6D–6F). We found a similarly strong growth defect when fully activating the Pho regulon by replacing the wild-type *PHO4* allele with a constitutively fully activated allele of *PHO4* (Springer et al., 2003) (Figures 6D–6F). We conclude that sequential activation of Pho genes is associated with a differential fitness cost: activation of the first wave of comes at a low fitness cost, whereas expression of second wave genes is significantly more deleterious when induced in rich media.

DISCUSSION

We describe a biphasic response to phosphate depletion in the budding yeast: an initial set of genes is induced immediately upon transfer to media lacking phosphate, whereas a second induction wave follows about 2 hr later (Figure 7A). The initial expression wave introduces a positive feedback loop that stabilizes the response, so that cells maintain this induction pattern stable even if phosphate is replenished. This stability is elimi-

nated with the induction of the second wave, which introduces an effective negative feedback loop.

This temporal order of gene induction is the key to its stability: when preventing the delay in induction of the second wave, the system oscillates and transiently downregulates the induced genes following their rapid induction. This reflects the destabilizing effects of the secondary response, and, in particular, the induction of the internal phosphatases *PHO8*, *PHM8*, and *PHM6*, which likely increase the available internal phosphate. The inhibition of ribosome synthesis, which also occurs during this time, eliminates the biggest drain on phosphate from the intracellular space and may further contribute to the negative feedback aspect of this second wave.

The two temporally separated responses differ not only in their effective feedback properties, but also in the extent to which they interfere with other cellular processes. The initial phase is rather mild; genes induced at this stage mostly retrieve phosphate from the external environment or from the dedicated phosphate storage (PolyP). In contrast, the second phase is

Figure 5. Pho Regulon Induction Precedes Cellular Stress in Mutants that Delay Pho Induction

(A) Gene expression pattern in mutants showing delayed activation. The time-resolved transcription profiling described in Figure 1 above was repeated for the indicated strains. Shown is the expression of Pho genes (left), stress genes (middle), and ribosome-associated genes (right). Vertical lines mark the maximal change in expression of the Protein synthesis module for the wild-type (black) $\Delta vip1$ (blue) and *pho90c* (gray). (B) Delay induction of the stress response. Maximal change in expression was calculated for the stress and protein synthesis predefined modules. Color code as in (A); black circles marking the maximal change in expression. (C) Pho gene induction precedes stress the response. Response time of the Pho genes was quantified as described in Figure 1E above and is shown for the respective mutants (Experimental Procedures). (D) Growth rate correlates with stress response. Growth rate in medium lacking phosphate was measured as described in Figure 4D for the indicated mutants.

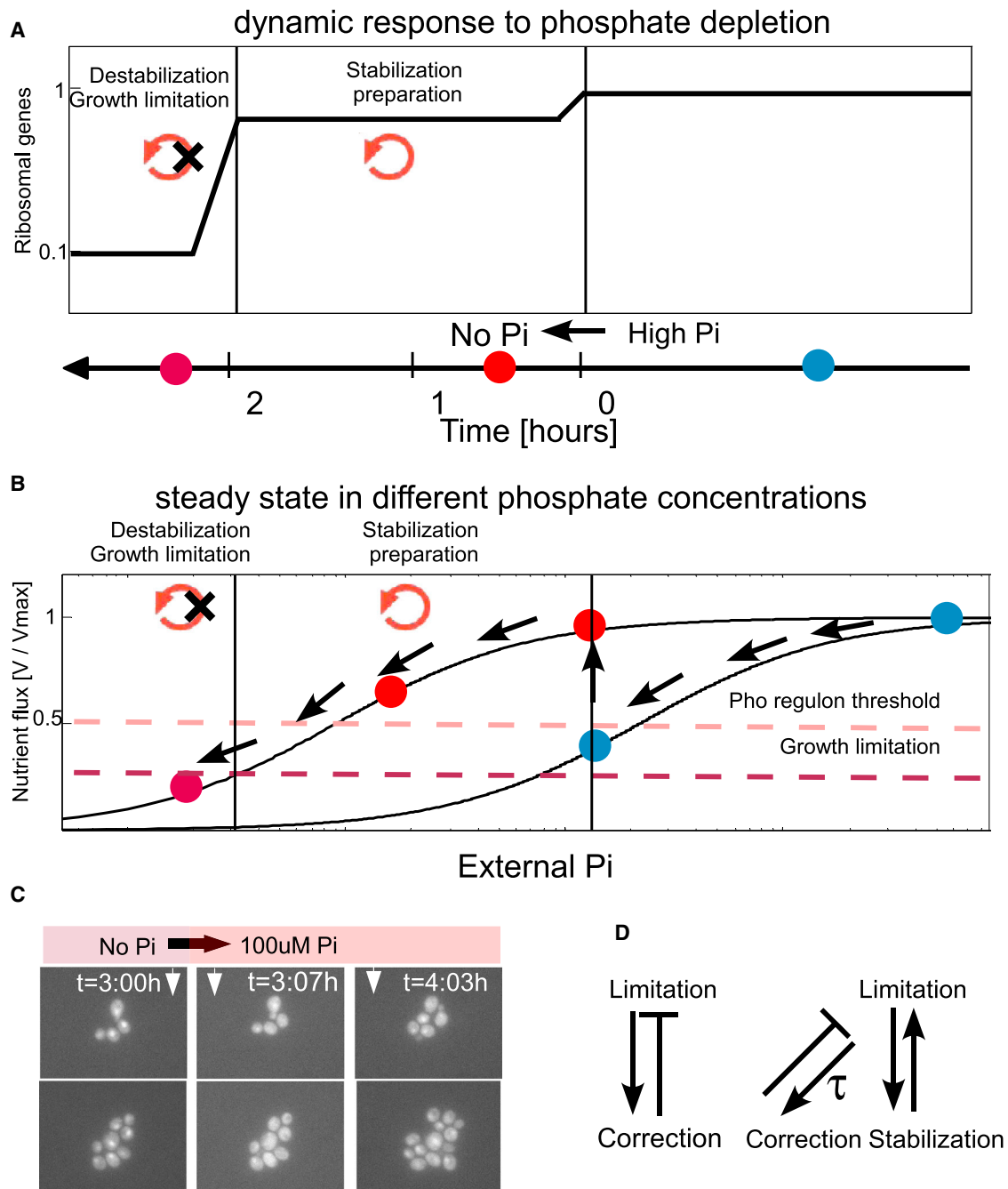


Figure 7. Steady-State Responses in Different Pi Concentrations Corresponds to Dynamic Response to Complete Pi Depletion

(A) Dynamic response to Pi depletion. When cells are transferred to a medium lacking phosphate, a subset of the Pho regulon genes are induced immediately (red circle), together with a slight reduction in the level of the ribosomal genes. After about 2 hr, a wave of delayed Pho genes is being activated and act as a negative feedback by releasing Pi from cellular substrates. Ribosomal genes expression is reduced about 10-fold compared to $t = 0$ and growth rate is reduced (deep red circle).

(B) Steady-state activation of the phosphate starvation response different Pi concentrations. The Pho regulon is induced as part of the starvation program when extracellular phosphate levels decrease to below the dissociation constant of the low-affinity transporter $\sim K_d$ ($\sim 220 \mu\text{M}$). This induction increases phosphate uptake back to its maximal velocity due to the increased expression of the high-affinity transporter Pho84 (blue circle to red circle). A positive feedback is therefore required to stabilize the starvation program in this regime. As Pi level drops further, the flux through the high-affinity transporters is decreasing and below the low-affinity transporters K_d ($\sim 220 \mu\text{M}$), and a secondary response is being activated, destabilizing the positive feedback and growth is limited (deep red circle).

(legend continued on next page)

more severe and includes also intracellular phosphatases that recycle phosphate from cellular components that may interfere with other cellular functions (Donella-Deana et al., 1993; Reddy et al., 2008; Xu et al., 2013). The separation between the two programs allows gradual adaptation to phosphate depletion: activation of a milder response may be sufficient under certain conditions (e.g., when some phosphate can still be retrieved from the media), whereas the more severe approach with its associated consequences is activated only if this initial program is not sufficient.

During the first 2 hr of starvation, replenishing phosphate does not downregulate Pho gene expression (Figure 7A). We previously described a similar commitment in steady-state continuous culture growing in medium with intermediate phosphate levels (Figure 7B) (Vardi et al., 2013). We suggest that in both cases, commitment results from the same feedback loop and, in particular, from the induction of the proteins Pho81 and Spl2; in the case of intermediate phosphate, the first induction wave retrieves internal phosphate to sufficient levels, alleviating the induction of the second, destabilizing wave. This destabilizing response is induced when steady-state cells grow in lower phosphate, conditions in which commitment is lost. Consistent with this hypothesis, we observe that transferring cells from low to intermediate phosphate results in the transient exit of Pho4 from the nucleus, prior to its re-entry (Figure 7C). This transient inactivation, in conditions where Pho4 is typically active, is a strong indication of the destabilizing effects introduced by the second wave genes.

More generally, correction mechanisms activated by cells to overcome limitations in nutrients or other factors have an inherent destabilizing effect: by reducing an intracellular limitation they counteract the process that led to their induction in the first place. This may be beneficial in some cases, when the limitation is transient. However, maintaining stable expression may be advantageous when cells are faced with a continuous limitation. The temporal strategy we describe here, whereby a stabilizing, mild response precedes the induction of a more severe destabilizing program, presents one strategy for ensuring a stable response under such conditions (Figure 7D).

EXPERIMENTAL PROCEDURES

Strains and Media

Strains were grown in synthetic complete (SC) medium, except of phosphate in the form of KH₂PO₄ that was added separately in different concentrations as described in the main text. The level of potassium was preserved by adding KCl instead of KH₂PO₄ in proper amount. The media pH was set to 5. Media for chemostat experiments included also 500 μ l/l of antifoam (Sigma-Aldrich A5758).

All yeast strains used in this study were constructed on a BY4741 background (MATa his3- Δ 1 leu2- Δ 0 met15- Δ 0 ura3- Δ 0) using standard genetic methods and are listed in Table S1. All strains were validated by PCR and sequencing of the relevant fragments. For a full list of strains, see the Supplemental Information.

Flow Cytometry

FACS analysis of PHO84pr-YFP was done by BD LSRII system (BD Biosciences). Flow cytometry was conducted with excitation at 488 nm and emission at 525 \pm 25 nm for GFP samples. For mCherry markers excitation was conducted at 594 nm and emission at 610 \pm 10 nm.

RNA Extraction and Sequencing

Samples were frozen in liquid nitrogen and RNA was extracted using Nucleospin 96 RNA kit Cells lysis was done in a 96-well plate by adding 450 μ l of lysis buffer containing 1 M sorbitol (Sigma-Aldrich), 100 mM EDTA, and 0.45 μ l lyticase (10 IU/ μ l). The plate was incubated in 30°C of 30 s in order to break the cell wall and then centrifuged for 10 s at 2,500 rpm, and the soup was transferred to a new 96-well plate, that provided by the Nucleospin 96 RNA kit. From that stage on, the extraction continued using this kit. From RNA extracts, cDNA was made for each sample. The cDNA of each samples were run in the Illumina highseq 2500.

RNA Sequencing Analysis

RNA reads were aligned to the yeast strain S288C R64 reference genome with BOWTIE. Number of reads for each gene was normalized by the total number of reads and multiplied by 10⁶. Genes that obtained below ten reads were discarded from the analysis. After alignment to the genome read number per sample was calculated samples that had less than 5 \times 10⁵ reads were discarded from the analysis in order to prevent an artificial enrichment for highly expressed genes. The expression at those time points was calculated as the mean between the two closest time points in the time course.

Calculation of Response Times for Different Genes

RNA expression data were smoothed with MATLAB cubic smoothing spline. Derivative was calculated at each point of the smoothed function, and the response time was taken as the first local maxima of the derivative (peak height >2). If the derivative at the first time point was higher than two, response time for those genes was determined as the first point in the time course.

Quantifying Oscillations of the Pho Regulon Genes

Pho regulon genes were divided in to two groups based on the response time calculated previously. Mean expression dynamics was smoothed using MATLAB cubic smoothing spline, and local minimums and maximums were calculated for the smoothed function. An oscillatory behavior was defined if expression fold change at local minima was above 10% compared to the local maximums surrounding it.

Calculation Induction Time of Expression Modules

Mean fold change was calculated for the genes in each module during the time course. The data were smoothed as described previously, and the maximal change in expression was defined as the induction time of the module.

Growth Rate

Cells were grown in high Pi to optical density (OD) = \sim 0.1 and then washed and transferred to no Pi medium. OD measurements were taken every 15 min. Growth rates and the time of switch between them were quantified by fitting the data to two exponential curves. Two repeats and the fit to one of the repeats are shown. Fit parameters are shown in Table S2.

Competition Assays

Cells were grown to logarithmic phase in SC medium (OD \sim 0.5) and washed in the relevant medium. Wild-type (WT) Reference (haploid and diploidB) and WT mCherry (haploid and diploid) strains were coinoculated in the specified media in 30°C. The WT-mCherry initial frequency was \sim 50%. Generation times were calculated from the measured OD: number of generations = log2(final

(C) Transient deactivation of the Pho regulon upon switching from no Pi to intermediate Pi. Logarithmic grown cells were washed grown in a CellASIC microfluidic imaging device. Nuclear localization of Pho4-GFP reporter before, during, and after a shift from medium lacking phosphate to 100 μ M Pi medium.

(D) Two models to the activation of a starvation response. In the simple model, the starvation signal activates a correction mechanism that may act as a negative feedback on the response (left). A second model suggests that a positive feedback is first induced by the starvation response, and, only after a delay of τ , the correction mechanism is being activated (right).

OD / initial OD). The relative frequencies of WT-mCherry and WT-Reference were measured by flow cytometry. The design of the cells dilution timescales was set from the cells growth rate in the specified condition, and the desired final OD. Specifically, cells were diluted once a day ($\Delta pho80$, $\Delta pho80 \Delta pho4$, EB1264, and EB1265 experiments) in order to reach a stationary phase, or twice a day (WT-induced versus WT uninduced experiment) in order to maintain cells in logarithmic phase. A linear fit for the logarithm of the WT frequency dynamics was used to calculate the slope for each competition assay. The relative WT fitness advantage is calculated from the slope divided by $\log(2)$. As a control, competition assays were done between similar strains expressing (WT-mCherry) and not expressing (WT-reference) a fluorescence marker, and no fitness advantage was detected. The control was repeated in different phosphate concentrations.

Microscopy

Cells were grown in high Pi medium over night to log phase, after which they were washed and suspended in medium lacking phosphate for 3 hr. Cells were transferred to a CellASIC microfluidics device (YC4D plates, <http://www.cellasic.com>) and grown in medium lacking phosphate for additional 3 hr, before medium was shifted to 100 μ M Pi medium. Cells were followed with an Olympus IX-81-ZDC inverted microscope with a motorized stage and autofocus ability and images were taken using every 7 min Image sets were acquired with a Hamamatsu ORCA-II-BT camera using a plan-apo 60 \times air objective.

ACCESSION NUMBERS

RNA sequencing data have been deposited to the NCBI Gene Expression Omnibus and are available under accession number GSE61668.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.10.002>.

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