Inositol pyrophosphate dynamics reveals control of the yeast PHO starvation program through 1,5-IP_8 and the SPX domain of Pho81

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Abstract

Eukaryotic cells control cytosolic inorganic phosphate to balance its role as essential macronutrient with its negative bioenergetic impacts. Phosphate homeostasis depends on a conserved signaling pathway including inositol pyrophosphates (PP-IPs) and SPX receptor domains. Since cells synthesize various PP-IPs and SPX domains bind them promiscuously, it is unclear whether a specific PP-IP regulates SPX domains in vivo, or whether multiple PP-IPs act as a pool. In contrast to previous models, which postulated that phosphate starvation is signaled by increased 1-IP_7 production, we now show that the levels of all detectable PP-IPs of yeast, 1-IP_7, 5-IP_7 and 1,5-IP_8, strongly decline upon phosphate starvation. Among these, specifically the decline of 1,5-IP_8 triggers the transcriptional phosphate starvation response, the PHO pathway. 1,5-IP_8 inactivates the cyclin-dependent kinase inhibitor Pho81 through its

SPX domain. This stimulates the cyclin-dependent kinase Pho85/Pho80 to phosphorylate the transcription factor Pho4 and repress the PHO pathway. Combining our results with observations from other systems we propose a unified model where 1,5-IP_8 signals cytosolic phosphate abundance to SPX proteins in fungi, plants, and mammals. Its absence triggers starvation responses.

Significance statement

Cytosolic P_i is of prime importance for cellular bioenergetics because P_i influences free energy of nucleotide hydrolysis and the metabolite fluxes through glycolysis and oxidative phosphorylation. Eukaryotic cells signal P_i via SPX domains binding critical ligands, inositol pyrophosphates (IP_7, IP_8), which control P_i homeostasis through a network of target proteins that import, export, store or detoxify P_i. Studies with different systems failed to yield a coherent model on this regulation.

We performed the first time-resolved profiling of the full isomer spectrum of inositol pyrophosphates and dissected the isomer that is relevant to intracellular P_i signaling. Our results support a unified model of P_i signaling across all eukaryotic kingdoms, which is in accord with the fundamental importance of P_i management for metabolism.

Introduction

Inorganic phosphate (P_i) is an essential nutrient for all living systems. While required in large amounts for synthesis of nucleic acids, phospholipids and phosphorylated carbohydrates and proteins, an overaccumulation of P_i decreases the free energy provided by P_i-liberating reactions, such as nucleotide hydrolysis, which might stall metabolism (1). In fungi, plants and animals, control of P_i homeostasis involves *myo*-inositol pyrophosphates (PP-IPs) and a family of evolutionarily conserved SPX domains (2).

SPX domains interact with or form part of a large variety of proteins that affect P_i homeostasis by transporting P_i across membranes, converting it into polyphosphates (polyP), metabolizing it, or regulating P_i-dependent transcription (3). Direct regulation of an SPX-containing protein by synthetic inositol pyrophosphates was shown for the polyP polymerase VTC, the P_i transporters Pho91 and the PHR transcription factors in plants (4–10). A firm link suggesting the SPX domain as the receptor for inositol pyrophosphate regulation was provided by point mutants in the SPX domain that rendered VTC either independent of activation by inositol pyrophosphates, or non-responsive to inositol pyrophosphates (6). Structural analysis revealed that many of these mutations localized to a highly charged region that can bind inositol poly- and pyrophosphates with high affinity. However, this binding site discriminates poorly between different inositol polyphosphates at the level of binding (6). By contrast, strong differences are observed in the agonist properties, leading to the suggestion that binding affinity is a poor predictor of inositol pyrophosphate specificity and activity (1, 4).

PP-IPs that can be found in a wide variety of organisms are 5-IP_7, 1-IP_7 and 1,5-IP_8. They are all linked to phosphate homeostasis by the fact that genetic ablation of the enzymes making them, such as IP6Ks, PPIP5Ks and IPTKs, alter the phosphate starvation response. Considerable discrepancies exist in the assignment of these PP-IPs to different aspects of P_i homeostasis. In mammalian cells, IP_8 activates the only SPX-protein expressed in this system, the P_i exporter XPR1 (11, 12). In plants, IP_7 and IP_8 decrease upon P_i starvation and mutants lacking either of the enzymes necessary for their synthesis induce the phosphate starvation response, leaving it open whether IP_7, IP_8, or both, control the P_i starvation program (10, 13, 14). Pioneering studies on the regulation of the P_i starvation program of S. cerevisiae, the PHO pathway,

concluded that this transcriptional response is triggered in by an increase in 1-IP_7 (15, 16), whereas subsequent studies in the pathogenic yeast Cryptococcus neoformans proposed 5-IP_7 as the necessary signaling compound (17). Studies using the S. cerevisiae polyphosphate polymerase VTC as a model suggested that this enzyme, which is necessary for polyP accumulation under P_i-replete conditions, is stimulated by 5-IP_7 in vivo (4, 18, 19), whereas studies in S. pombe proposed IP_8 as the stimulator (20). S. pombe has similar enzymes for IP_8 synthesis and hydrolysis as S. cerevisiae (21–25) In contrast to S. cerevisiae, genetic ablation of 1-IP_7 and IP_8 production in S. pombe leads to hyper-repression of the transcriptional phosphate starvation response (23, 26, 27) and interferes with the induction of the transcriptional phosphate starvation response. However, the transcriptional phosphate starvation response in S. pombe organism occurs through a different set of protein mediators. For example, it lacks homologs of Pho81 and uses Csk1 instead of Pho80/Pho85 for phosphate-dependent transcriptional regulation (28–31). Furthermore, PHO pathway promotors in S. pombe overlap with and are strongly regulated by lncRNA transcription units (32–34). It is hence difficult to compare the downstream events in this system to the PHO pathway of S. cerevisiae.

The discrepancies mentioned above could either reflect a true divergence in the signaling properties of different PP-IPs in different organisms, in which case a common and evolutionarily conserved signaling mechanism may not exist. Alternatively, the diverging interpretations could reflect limitations in the analytics of PP-IPs and in the in vivo assays for the fundamental processes of P_i homeostasis. The analysis of PP-IPs is indeed very challenging in numerous ways. Their cellular concentrations are very low, the molecules are highly charged, and they exist in multiple isomers that differ only in the positioning of the pyrophosphate groups. PP-IP analysis has traditionally been performed by ion exchange HPLC of extracts from cells radiolabeled through ^{3}H-inositol (35). This approach requires constraining and slow labeling schemes, is costly and time-consuming. Furthermore, HPLC-based approaches in most cases did not resolve isomers of IP_7 or IP_8. These factors severely limited the number of samples and conditions that could be processed and the resolution of the experiments.

The recent use of capillary electrophoresis coupled to mass spectrometry (CE-MS) has dramatically improved the situation, permitting resolution of many regio-isomers of IP_7 and IP_8 without radiolabeling, and at superior sensitivity and throughput. We harnessed the potential of this method to dissect the role of the three known PP-IPs that accumulate in S. cerevisiae. We analyzed their impact on the PHO pathway, which is a paradigm for a P_i-controlled transcriptional response and the regulation of phosphate homeostasis (1, 36, 37). Beyond this physiological function, however, the PHO pathway also gained widespread recognition as a model for promotor activation, transcription initiation, chromatin remodeling and nucleosome positioning (38). In the PHO pathway, the cyclin-dependent kinase inhibitor (CKI) Pho81 translates intracellular P_i availability into the activation of the cyclin-dependent kinase (CDK) complex Pho80-Pho85 (15, 39–41). At high P_i, Pho80-Pho85 phosphorylates and inactivates the key transcription factor of the PHO pathway, Pho4, which then accumulates in the cytosol (42–44). During P_i starvation, Pho81 inhibits the Pho80-Pho85 kinase, leading to dephosphorylation and activation of Pho4 and the ensuing expression of P_i-responsive genes (PHO genes).

The activation of Pho80-Pho85 through Pho81 has been explored in detail, leading to a series of highly influential studies that have gained wide acceptance in the field. These studies ascribed a critical function to 1-IP_7 in activating the CDK inhibitor PHO81, allowing it to inhibit Pho80-Pho85 (15). They reported an increase of IP_7 concentration upon P_i starvation and concluded that this increase was necessary and sufficient to inactivate Pho80/Pho85 kinase through Pho81 and trigger the PHO pathway. The 1-IP_7 binding site on Pho81 was mapped to a short stretch of 80 aa, the "minimum domain" (15, 16, 40, 45). This domain is in the central region and distinct from the N-terminal SPX domain of Pho81. Since overexpression of the minimum domain rescued P_i-dependent regulation of the PHO pathway to some degree, the key regulatory function was ascribed to this minimum domain and the SPX domain was considered as of minor importance. By contrast, earlier studies based on unbiased mutagenesis, which subsequently received much less attention, had identified mutations in other regions of PHO81 with significant impact on PHO pathway activation (40, 46–49). The situation is complicated by further studies, which found global IP_7 levels to decrease rather than increase upon P_i starvation (6, 11, 18). The analytics used could not distinguish 1-IP_7 and

5-IP_7, however, leaving open the possibility that an increase in 1-IP_7 might be masked by a decrease in the much larger pool of 5-IP_7.

We capitalized on recent advances in the non-radioactive analysis of inositol pyrophosphates through CE-MS, offering superior resolution, sensitivity, and throughput (50, 51), to revisit the regulation of the PHO pathway by PP-IPs and Pho81. To this end, we combined comprehensive analyses of PHO pathway activation and PP-IP profiles in mutants of all key enzymes involved in PP-IP metabolism of yeast.

Results

So far, analysis of the role of PP-IPs in P_i homeostasis and P_i starvation responses relied mainly on the use of mutants which ablate one of the pathways of PP-IP synthesis (Fig. 1). Yet, as shown in plants, several PP-IPs can change in a similar manner upon P_i depletion or replenishment, and ablation of enzymes diphosphorylating either the 1- or 5-positions on the inositol ring induces similar P_i starvation responses (10, 13) This renders it difficult to distinguish a role for an individual PP-IP from the alternative hypothesis that all PP-IPs collectively contribute to signaling. To dissect this issue in yeast, we performed time course analyses of mutants in PP-IP phosphatases and kinases, in which we correlate the levels of all PP-IPs with the induction of the phosphate starvation response in yeast. The rationale was to seek for upper or lower thresholds of PP-IP concentrations during PHO pathway induction and using those to sieve out the PP-IP responsible for signaling P_i starvation.

Cytosolic concentrations and kinetics of 5-IP_7, 1-IP_7 and 1,5-IP_8

In yeast, the *myo*-inositol hexakisphosphate kinases Vip1 and Kcs1 generate 1-IP_7 and 5-IP_7, respectively, and they are both required for synthesis of 1,5-IP_8 (Fig. 1) (52–56). Inositol pyrophosphatases, such as Ddp1 and Siw14 dephosphorylate these compounds at the 1- and 5 position, respectively (Fig. 1A) (18, 57, 58).

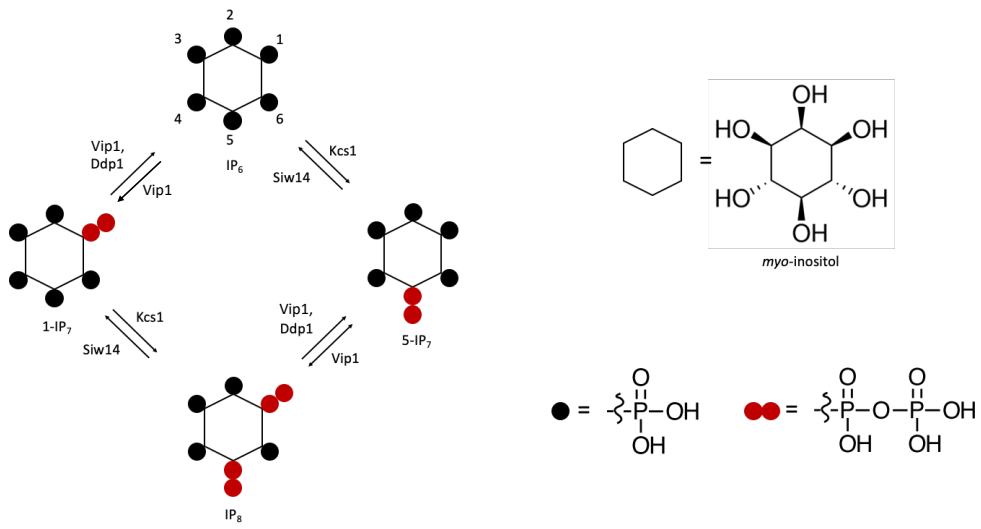


Fig. 1: Pathways of inositol pyrophosphate metabolism in *S. cerevisiae*.

We analyzed the kinetics and the role of these inositol pyrophosphates in PHO pathway activation. To this end, yeasts were cultured in synthetic liquid (SC) media to early logarithmic phase and then transferred to P_i-free SC medium. The cells were extracted with perchloric acid and inositol phosphates were analyzed by capillary electrophoresis coupled to electrospray ionization mass spectrometry (50). Three PP-IPs were detectable: 1-IP_7, 5-IP_7 and 1,5-IP_8. We note that the CE-MS approach does not differentiate pyrophosphorylation of the inositol ring at the 1- and 3-positions. Thus, our assignments of the relevant species as 1-IP_7 and 1,5-IP_8 are based on previous characterization of the reaction products and specificities of IP6Ks and PPIPKs (52–56, 59).

Quantitation of PP-IPs by CE-MS was facilitated by spiking the samples with synthetic, ^{13}C-labeled inositol pyrophosphate standards (60, 61). The recovery rate of inositol pyrophosphates during the extraction was determined by adding known quantities of synthetic standards to the cells already before the extractions. This demonstrated that 89 % of 1-IP_7, 90 % of 5-IP_7 and 75 % of 1,5-IP_8 were recovered in the extract. To estimate the cellular concentrations of these compounds the volume of the cells was determined by fluorescence microscopy after staining of the cell wall with trypan blue (Fig. S1). This yielded an average cell volume of 42 fL. Detailed morphometric studies of yeast showed that the nucleus occupies around 8% of this volume and that all other organelles collectively account for approx. 18%

(62). Taking this into account we can estimate the concentrations in the cytosolic space (including the nucleus, which is permeable to small molecules) of cells growing logarithmically on SC medium as 0.3 µM for 1-IP_7, 0.7 µM for 5-IP_7, 0.5 µM for 1,5-IP_8 (Fig. 2A). IP_6 is present at 3 µM.

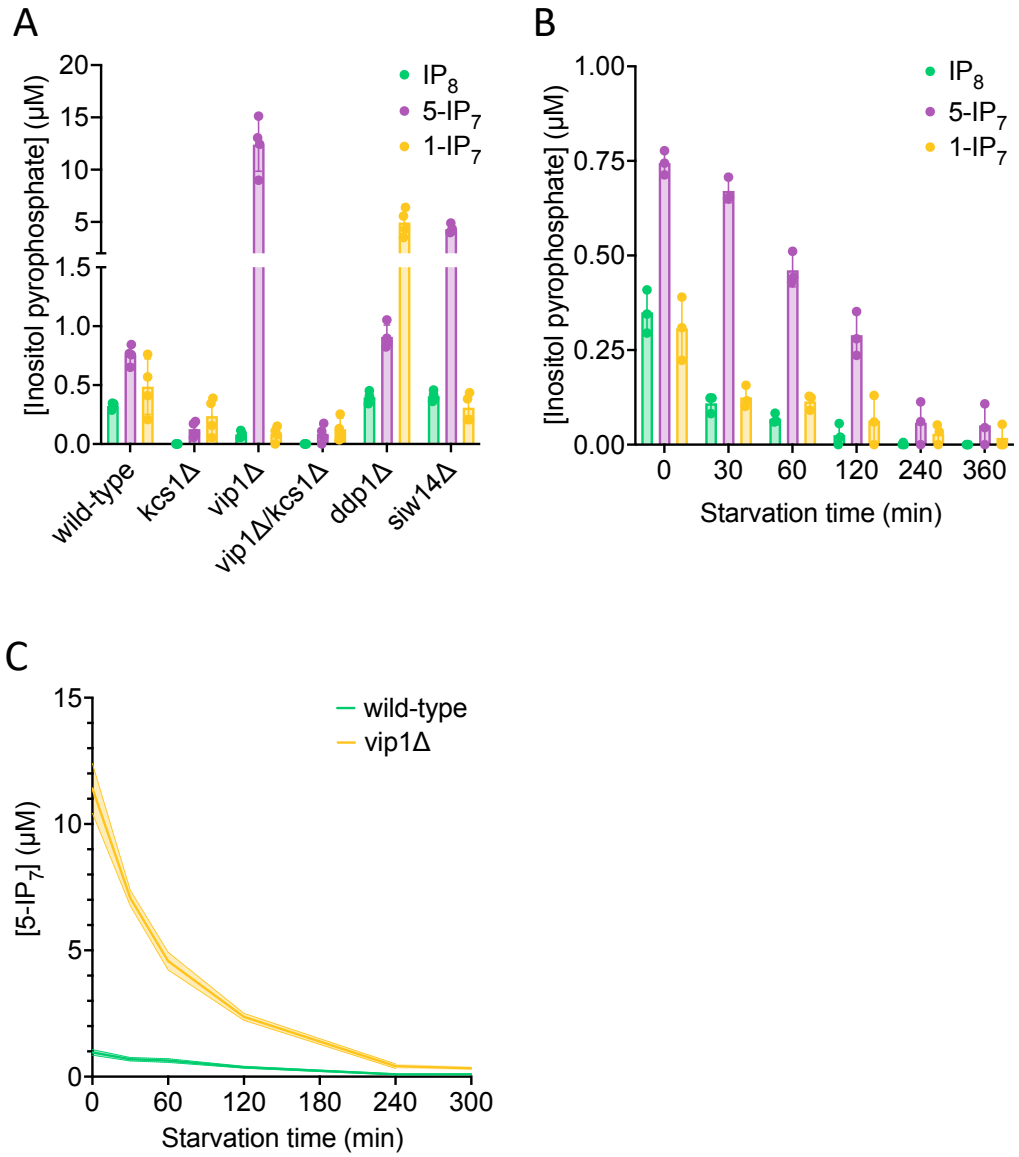


Fig. 2. Cytosolic concentrations of 5-IP_7, 1-IP_7 and IP_8.

(A) Inositol pyrophosphate concentrations in the cytosol. The indicated strains were grown logarithmically in SC medium containing 7.5 mM of P_i (30°C, 150 rpm, overnight). When OD_{600nm} reached 1 (1 x 10^7 cells/ml), 1 ml of culture was extracted with perchloric acid and analyzed for PP-IPs by CE-ESI-MS. The y-axis provides the estimated cytosolic concentrations based on an average cell volume of 42 fL. Means (n=4) and standard deviations are indicated.

(B) Evolution of Inositol pyrophosphate species during P_i starvation. Cells were grown as in A, washed twice with P_i starvation medium and further incubated in P_i starvation medium. The inoculum for the samples bound to be extracted after different times of further incubation in starvation medium was adjusted such that all samples had similar OD_{600nm} at the time of harvesting (OD_{600nm}=0.5 for 30 min

and 60 min samples; OD_{600nm}=0.4 for 120 min and 240 min samples; OD_{600nm}=0.25 for 360 min samples). At the indicated times in starvation media, 1 ml aliquots were extracted analyzed for PP-IPs as in A. The data was normalized by the number of cells harvested before calculating cytosolic concentrations. Means and standard deviations are given (n=3).
(C) Depletion of 5-IP_7 in starving *vip1Δ* cells. The indicated cells were grown in Pi-replete medium and then transferred to Pi starvation medium as in B. At the indicated times, samples were extracted and analyzed for 5-IP_7 as in A. Means and standard deviations (n=4) are shown as solid lines and shaded areas, respectively.

Next, we determined the impact of Kcs1, Vip1, Siw14 and Ddp1 on the inositol pyrophosphate levels in the cells (Fig. 2A). 5-IP_7 has not been detected in the *kcs1Δ* mutant and 1-IP_7 has been strongly reduced in the *vip1Δ* strain. IP_8 was undetectable in *kcs1Δ* and decreased by 75% in *vip1Δ*. *kcs1Δ* mutants also showed a 2 to 3-fold decrease in 1-IP_7, suggesting that the synthesis of 1-IP_7 depends on 5-IP_7. This might be explained by assuming that a significant source of 1-IP_7 is synthesis of 1,5-IP_8 through successive action of Kcs1 and Vip1, followed by dephosphorylation to 1-IP_7. A systematic analysis of this interdependency will require pulse-labeling approaches, which are not yet available for inositol pyrophosphates. Therefore, this aspect was not pursued further in the framework of this study. An unexpected finding was the up to 20-fold overaccumulation of 5-IP_7 in the *vip1Δ* mutant. By contrast, *ddp1Δ* cells showed normal levels of 5-IP_7 and 1,5-IP_8 but a 10-fold increase in 1-IP_7. *siw14Δ* cells showed a 5-fold increase in 5-IP_7, but similar levels of 1,5-IP_8 and 1-IP_7 as wildtype.

We performed kinetic experiments to analyze how PP-IP levels change under P_i withdrawal. 5-IP_7 was the predominant inositol pyrophosphate species in wildtype cells growing on P_i-replete media (Fig. 2B). Within 30 min of P_i starvation, the concentration of all three inositol pyrophosphate species rapidly decreased by 75% for 1,5-IP_8, by 47% for 1-IP_7, and by 40% for 5-IP_7. This decline continued, so that 1-IP_7 and 1,5-IP_8 became undetectable and only 3% of 5-IP_7 remained after 4 h, corresponding to a concentration below 50 nM. The high excess of 5-IP_7 in *vip1Δ* cells also declined as soon as the cells were transferred to P_i starvation medium (Fig. 2C). After two hours of starvation, it was still two-fold above the concentration measured in P_i-replete wildtype cells. Even after 3.5-4 h, P_i-starved *vip1Δ* cells had just reached the 5-IP_7 concentration of P_i-replete wildtype cells.

Taken together, P_i starvation leads to a virtually complete depletion of all three inositol pyrophosphate species, with 1,5-IP_8 reacting faster than 1-IP_7 and 5-IP_7. Furthermore, inositol pyrophosphatase mutants provide the possibility to generate relatively selective increases in 5-IP_7 and 1-IP_7. We used this information to dissect the impact of 5-IP_7, 1-IP_7 and 1,5-IP_8 on control of the PHO pathway.

1,5-IP_8 signals cytosolic P_i levels to the PHO pathway

To this end, we correlated the measured inositol pyrophosphate concentrations to the induction of the PHO pathway. We assayed a key event of PHO pathway activation, partitioning of the fluorescently tagged transcription factor $Pho4^{yEGFP}$ between the cytosol and the nucleus. Pho4 shuttles between nucleus and cytosol and its phosphorylation through Pho85/Pho80 favors Pho4 accumulation in the cytosol. The relocation of Pho4 can hence serve as an in vivo indicator of PHO pathway activation (63–65). It provides a readout for Pho85/Pho80 activity. PHO4 was tagged at its genomic locus, making $Pho4^{yEGFP}$ the sole source of this transcription factor. Pho4 relocation was assayed through automated image segmentation and analysis. The artificial intelligence-based segmentation algorithm recognized more than 90% of the cells in a bright-field image and delimited their nuclei based on a red-fluorescent nuclear mCherry marker (Fig. S2). This segmentation allows quantitative measurements of Pho4 distribution between the cytosol and nucleus in large numbers of cells. In addition, we assayed PHO pathway activation through fluorescent yEGFP reporters expressed from the PHO5 (prPho5-yEGFP) and PHO84 (prPho84-yEGFP) promotors. These are classical assays of PHO pathway activation, but their output is further downstream and hence comprises additional regulation, e.g. at the level of chromatin or RNA, or the direct activation of Pho4 through metabolites such as AICAR (66–70). Upon P_i withdrawal, both promotors are induced by the PHO pathway but the PHO84 promotor reacts in a more sensitive manner and is induced more rapidly than the PHO5 promotor (63).

In wildtype cells grown under P_i-replete conditions, $Pho4^{yEGFP}$ was cytosolic and the PHO5 and PHO84 promotors were inactive, indicating that the PHO pathway was repressed (Fig. 3). Within 30 min of starvation in P_i-free medium, $Pho4^{yEGFP}$ relocated into the nucleus and PHO84

and PHO5 were strongly induced. By contrast, *kcs1Δ* cells showed Pho4^{yEGFP} constitutively in the nucleus already under P_i-replete conditions, and PHO5 and PHO84 were activated. These cells have strongly reduced 1,5-IP_8 and 5-IP_7, and 50% less 1-IP_7 than the wildtype, Thus, a decline of PP-IPs not only coincides with the induction of the P_i starvation program, but the genetic ablation of these compounds is sufficient for a forced triggering of this response in P_i-replete conditions. Therefore, we explored the hypothesis that inositol pyrophosphates repress the PHO pathway, and that their loss upon P_i starvation creates the signal that activates the starvation response.

In this case, we must explain the behavior of the *vip1Δ* mutation, which strongly reduces 1-IP_7 and 1,5-IP_8 and maintains the PHO pathway repressed in P_i-replete medium. Upon withdrawal of P_i, *vip1Δ* cells did not show nuclear relocation of Pho4 after 30 min and, even after 4h of starvation, PHO5 was not expressed. PHO84 remained partially repressed in comparison with the wildtype. These results are at first sight consistent with the proposal that 1-IP_7 activates the PHO pathway (15, 16). Several further observations draw this hypothesis into question, however. First, all three inositol pyrophosphate species strongly decline upon P_i starvation instead of showing the increase postulated by Lee et al. Second, *vip1Δ* cells show a more than 15-fold overaccumulation of 5-IP_7. Genetic ablation of this pool by deleting KCS1 is epistatic to the *vip1Δ* mutation. A *kcs1Δ vip1Δ* double mutant constitutively activates the PHO pathway already in presence of P_i, despite its strong reduction of 1-IP_7 and the complete absence of 1,5-IP_8. Third, *ddp1Δ* cells, which show a 10-fold quite selective increase in 1-IP_7 under P_i-replete conditions, did not induce the PHO84 and PHO5 promotors, nor did they show Pho4 accumulation in the nucleus in P_i-replete medium. Thus, even a strong increase in 1-IP_7 is not sufficient to activate the PHO pathway, while reductions of inositol pyrophosphates do this.

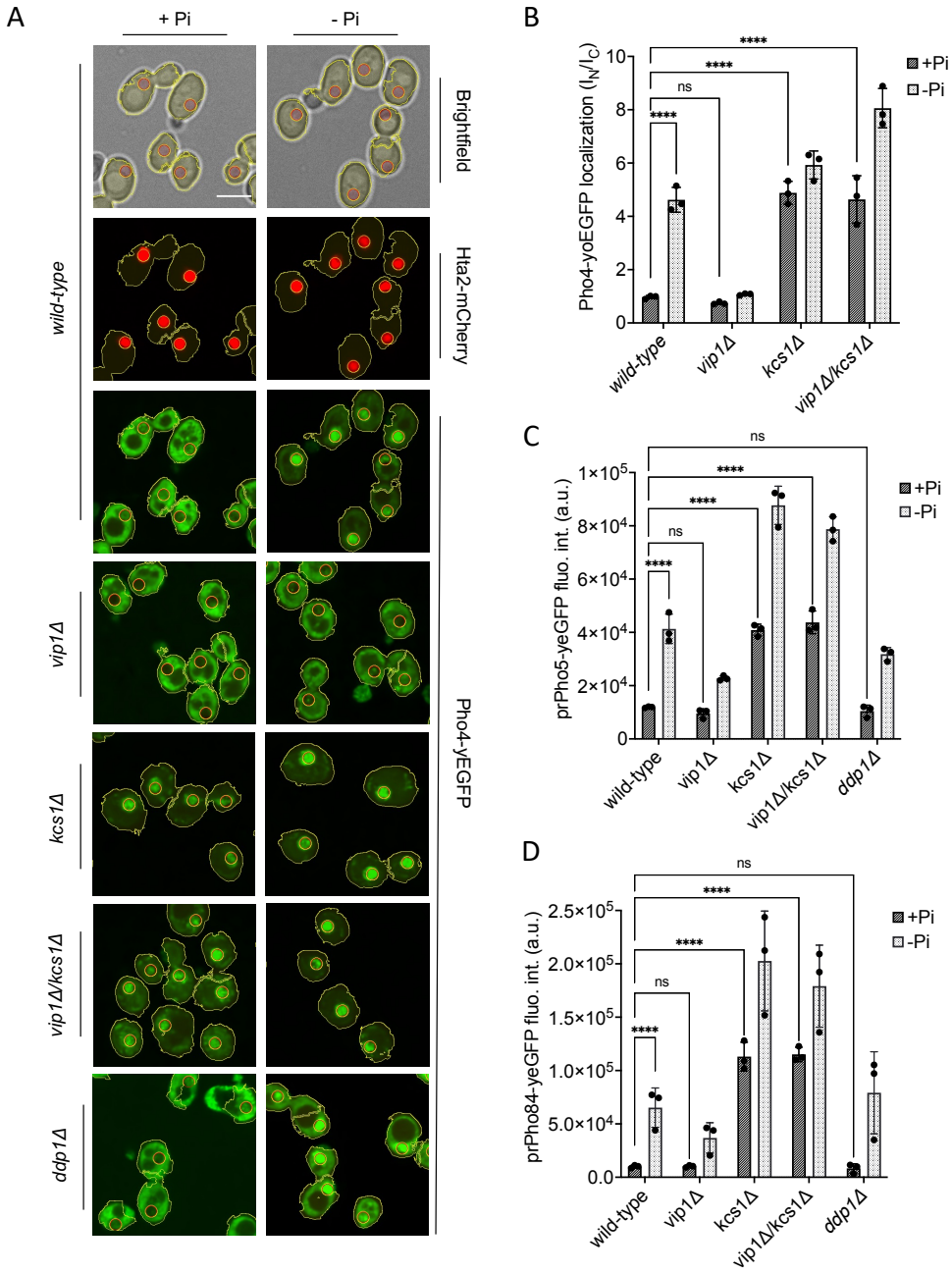


Fig. 3. Inhibition of the PHO pathway by excessive 5-IP$_7$.

The indicated cells expressing Pho4^{yEGFP} and the histone Hta2^{mCherry} as a nuclear marker were logarithmically grown in P_i-replete SC medium, washed, and transferred to P_i starvation medium as in Fig. 2A.

(A) Subcellular localization of Pho4^{yEGFP} was analyzed on a spinning disc microscope. Cells are shown in the presence of 7.5 mM of P_i (+ P_i) or 30 min after the shift to P_i starvation (- P_i) medium. Yellow lines surrounding the cells illustrate the segmentation performed by the algorithm that was used to quantify Pho4^{yEGFP} distribution in B. Scale bar: 5 µM. λ_{ex}: 470 nm; λ_{em}: 495-560 nm.

(B) Average intensity of Pho4^{yEGFP} fluorescence was determined by automated image segmentation and analysis. Pho4^{yEGFP} localization is quantified by the ratio of the average fluorescence intensities in the nucleus over the average fluorescence intensity in the cytosol (I_N/I_C). XXX cells were analyzed per condition and experiment. n=3. Means and standard deviation are indicated.

(C) Activation of the PHO5 promotor. Cells expressing the prPho5-yEGFP reporter construct from a centromeric plasmid were grown in Pi-replete medium (7.5 mM P_i) as in Fig. 2A, and then shifted to P_i starvation medium or kept in P_i replete medium. After 4 h of further incubation, fluorescence intensity of the same number of cells was measured in a Spectramax EM microplate reader. λ_{ex}: 480 nm; λ_{em}: 510 nm. n=3. Means and standard deviation are indicated. *
(D) Activation of the Pho84 promotor. Cells expressing the prPho5-yEGFP reporter construct from a centromeric plasmid were treated and analyzed as in C.
*** p<0.0001; *** p<0.001; ** p<0.01; n.s.: not significant

The data described above argues against a diminution of 1-IP_7 as a critical factor for induction of the PHO pathway because *vip1Δ* cells, which have virtually no 1-IP_7, do not constitutively activate the PHO pathway. By contrast, *vip1Δ kcs1Δ* double mutants, which have similarly low levels of 1-IP_7 but also strong reductions of 1,5-IP_8 and 5-IP_7, show constitutive PHO pathway activity (Fig. 3). We hence tested the hypothesis that a decline of 5-IP_7 might trigger the PHO pathway. To define the critical concentration at which this happens in *vip1Δ* cells, we assayed the induction of the PHO pathway over time (Fig. 4). While $Pho4^{yEGFP}$ was cytosolic in wild-type cells under high P_i supply, a large fraction of it rapidly relocated into the nucleus within the first 15 min of P_i starvation (Fig. 4 A,B). In *vip1Δ*, $Pho4^{yEGFP}$ relocation followed a sigmoidal curve. The nuclear/cytosolic ratio strongly increased after 1 h, reaching a plateau after 3 h. Thus, relocation was stimulated when the 20-fold exaggerated 5-IP_7 levels of the *vip1Δ* cells had declined below 5 µM (Fig. 2C), which is 6 to 7 times above the maximal concentration observed in P_i-replete wildtype cells. Since 5-IP_7 is the only PP-IP that exists in *vip1Δ* cells in significant amounts, this observation places the critical concentration of 5-IP_7 that can repress the PHO pathway at 5 µM. In P_i-replete wildtype cells, 5-IP_7 never reaches 5 µM (Fig. 2) and, therefore, 5-IP_7 is unlikely to be a physiological repressor of the PHO pathway in wildtype cells. The 5-IP_7-dependent PHO pathway repression in *vip1Δ* cells must then be considered as a non-physiological reaction resulting from the strong overaccumulation of 5-IP_7 in these cells.

This interpretation is also consistent with the phenotype of *siw14Δ* cells. Although these cells accumulate 5-IP_7, they accumulate it 4-fold less than *vip1Δ*, remaining below the 5 µM threshold where we should expect non-physiological repression of the PHO pathway. However, *siw14Δ* cells contain the same concentration of 1,5-IP_8 as the wildtype (Fig. 2A), degrade it upon P_i starvation as the wildtype, and relocalize $Pho4^{yEGFP}$ with similar kinetics as the wildtype (Fig. S4). Taken together with the evidence presented above, 1,5-IP_8 thus ends

up being the only PP-IP that correlates with the activation of the PHO pathway in all mutants and conditions. This points to 1,5-IP_8 as the controller of the PHO pathway.

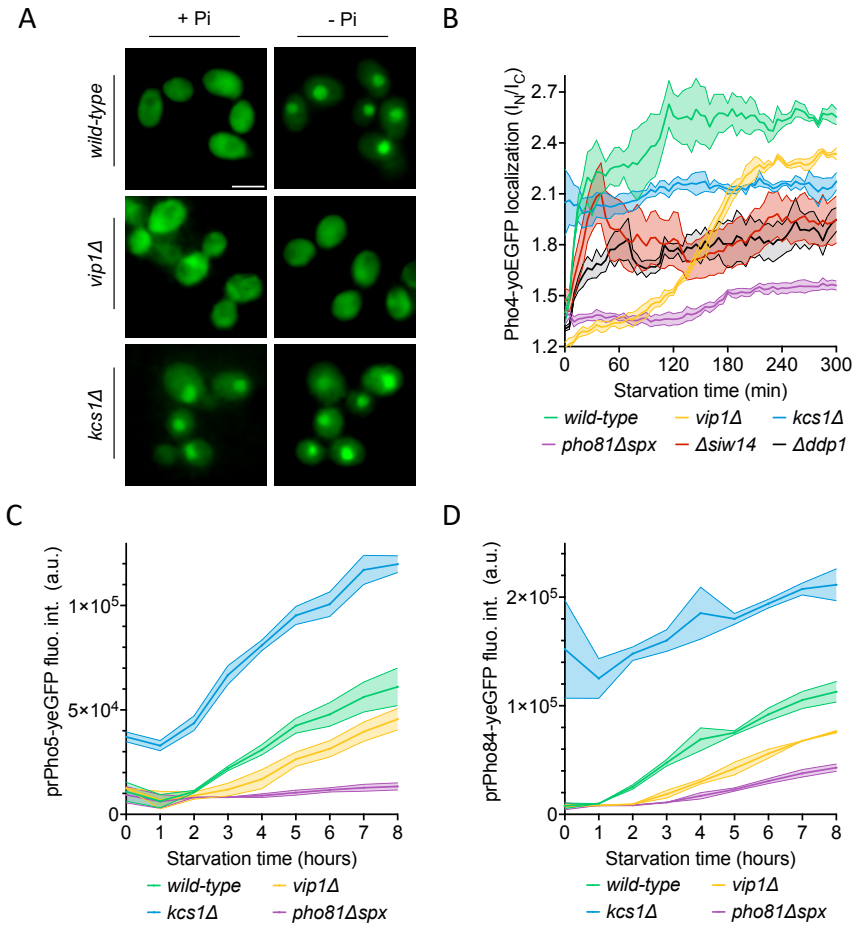


Fig. 4. Effect of Vip1, Kcs1 and Pho81 on the time course of Pho4 translocation and PHO-gene activation.

The indicated cells were logarithmically grown in P_i-replete SC medium, washed, and transferred to P_i starvation medium as in Fig. 2A. At the indicated times after transfer to Pi-starvation medium, they were analyzed for Pho4 localization, prPHO5-yEGFP expression, or prPHO84-yEGFP expression, using assays as in Fig. 3.

(A) Cells expressing $Pho4^{yEGFP}$ and the histone $Hta2^{mCherry}$ as a nuclear marker, shown in the presence of 7.5 mM of P_i (+ P_i) or after 90 min of P_i starvation (-P_i). Pictures were taken on a widefield fluorescence microscope equipped with a stage-top incubator (kept at 30°C) and an IBIDI flow chamber. Only the GFP-channel is shown. Scale bar = 5 µM. λ_{ex}: 470 nm; λ_{em}: 495-560 nm.

(B) Distribution of $Pho4^{yEGFP}$ between the nucleus and cytosol was quantified in the cells from A at various timepoints of P_i starvation. 100-200 cells were analyzed per condition and experiment at each timepoint. The solid lines and the shaded areas indicate the means and standard deviation, respectively.

(C) Activation of the PHO5 promotor. Cells expressing the prPho5-yEGFP reporter construct from a centromeric plasmid were grown in P_i-replete medium, shifted to P_i starvation medium, and analyzed for GFP fluorescence intensity (as in Fig. 3C) at the indicated timepoints of starvation. λ_{ex}: 480 nm; λ_{em}: 510 nm. n=3. The solid lines and the shaded areas indicate the means and standard deviation, respectively.
(D) Activation of the PHO84 promotor. As in C, but with cells expressing prPHO84-yEGFP as reporter.

The PHO pathway is controlled by the SPX domain of Pho81

Our results strongly argue against an increase of 1-IP_7 as the key factor for inactivating the CDK Pho80-Pho85 through the CDK inhibitor Pho81. This prompted us to also re-evaluate the regulation of Pho81 because previous studies had proposed a small peptide from the central region of Pho81, called the minimum domain (amino acids 645-724), as a receptor for 1-IP_7 that triggers the PHO pathway (15, 16). This model rests mainly on results with the isolated minimum domain used in vitro or, in highly overexpressed form, in vivo. This expression of the minimum domain outside its normal molecular context may be problematic because earlier work had suggested that the N- and C-terminal portions of Pho81 could provide competing inhibitory and stimulatory functions for Pho81 (40).

We targeted the N-terminal SPX domain of Pho81 through various mutations, leaving the rest of Pho81 and its minimum domain intact (Fig. 5). First, we asked whether Pho81 lacking the N-terminal 200 amino acids, corresponding to a deletion of its SPX domain, could still activate the PHO pathway. This $Pho81^{\Delta SPX}$ is likely to be folded because a yEGFP-tagged version of $Pho81^{\Delta SPX}$ localized to the nucleus (Fig. 5C, D), and this nuclear localization requires Pho81 to interact with Pho80-Pho85 (45). We tested the effect of $Pho81^{\Delta SPX}$ on relocation of $Pho4^{yEGFP}$ (Fig. 5 A, B). Whereas wild-type cells efficiently relocated $Pho4^{yEGFP}$ to the nucleus upon P_i starvation, *pho81*$^{\Delta SPX}$ cells partially maintained $Pho4^{yEGFP}$ in the cytosol. Thus, while the SPX domain is not essential for nuclear targeting of Pho81, it is required for efficient nuclear relocation of Pho4 and the induction of the PHO pathway under P_i starvation.

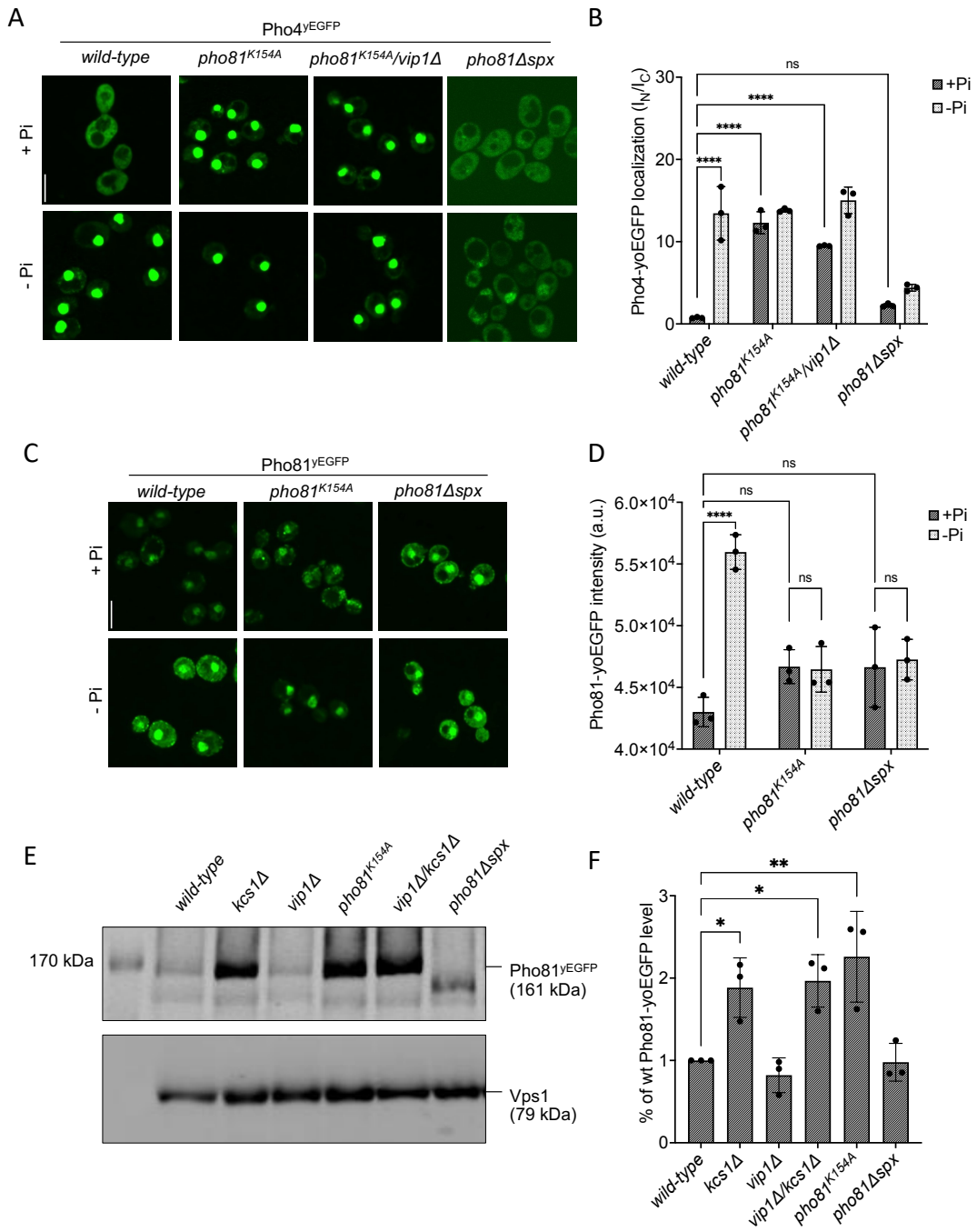


Fig. 5. SPX-dependent activation of the PHO pathway.

Cells were logarithmically grown in P_i-replete SC medium as in Fig. 2A, washed, and incubated for further 4 h in medium with 7.5 mM of P_i (+ P_i), or in starvation medium (- P_i).

(A) Pho4 relocation. The indicated cells expressed Pho4^{yEGFP} from its genomic locus. At the end of the 4 h starvation period, GFP fluorescence was imaged on a spinning disc microscope. Scale bar: 5 µm. λ_{ex}: 488 nm; λ_{em}: 500-530 nm.

(B) Quantification of the nuclear localization of Pho4^{yEGFP}. Images from A were subjected to automated segmentation and quantification of the average fluorescence intensity in the nucleus and cytosol as in Fig. 3B. 100-200 cells were quantified per sample. n=3 experiments.

(C) Pho81^{yEGFP} localization and expression. The cells expressed the indicated variants of Pho81^{yEGFP} from its genomic locus. At the end of the 4 h growth period, GFP fluorescence was imaged as in A.

(D) Quantification of the total cellular fluorescence of Pho81^{yEGFP}. Images from C were subjected to automated segmentation and the average fluorescence intensity of the entire cells was quantified as in Fig. 3B. 100-200 cells were quantified per sample. n=3 experiments.
(E) Pho81^{yEGFP} expression assayed by blotting. Whole-cell protein extracts were prepared from cells expressing the indicated variants of Pho81^{yEGFP}, which had been grown in P_i-replete SC medium as in Fig. 2A. Proteins were analyzed by SDS-PAGE and Western blotting of Pho81^{yEGFP}. Vps1 was decorated as a loading control.
(F) Quantification of Pho81^{yEGFP} blotting. Bands from experiments as in E were quantified on a LICOR Odyssey infrared fluorescence imager. The signals from wildtype cells were set to 1. Means and standard deviations are shown. n=3. **** p<0.0001; *** p<0.001; ** p<0.01; * p<0.05.

Several lysines located on α-helix 4 of SPX domains form part of an inositol pyrophosphate binding patch (6). Substituting them by alanine severely reduces the affinity of the domains for inositol pyrophosphates and can thus mimic the inositol pyrophosphate-free state. We determined the corresponding lysines in Pho81 (Fig. S3), created a point mutation in the genomic PHO81 locus that substitutes one of them, K154, by alanine, and investigated the impact on the PHO pathway. Pho81$^{K154A\text{-}yEGFP}$ should be correctly folded because it concentrates in the nuclei of the cells, which requires its interaction with Pho80-Pho85. *pho81^{K154A}* cells constitutively activated the PHO pathway because Pho4^{yEGFP} accumulated in their nucleus already under P_i-replete conditions (Fig. 5A, B). This constitutive activation was also observed in a *pho81^{K154A} vip1Δ* double mutant, confirming that 1-IP_7 synthesis by Vip1 is not necessary for PHO pathway induction. Since, as shown above, *vip1Δ* cells over-accumulate 5-IP_7 20-fold and thereby repress the PHO pathway, this result underlines the efficiency of the K154A substitution in preventing PP-IP binding to the Pho81 SPX domain. Induction of the PHO pathway through SPX mutations is further illustrated by Pho81 expression, which itself is under the control of the PHO pathway (Fig. 5E, F). Western blots of protein extracts from cells grown under P_i-replete conditions showed increased levels of Pho81 in pho81^{K154A} cells, like those observed in other strains with constitutively active PHO pathway, such as *kcs1Δ* and *vip1Δ/kcs1Δ*. They were significantly higher than in cells that do not constitutively activate the PHO pathway, such as wildtype or *vip1Δ*. In sum, these results suggest that the PHO pathway is regulated through binding of PP-IPs to the SPX domain of Pho81.

Inositol pyrophosphate binding to the SPX domain labilizes the Pho81-Pho80 interaction

The effect of inositol pyrophosphates on PHO pathway activation could be further investigated exploiting Pho80 substitutions (*pho80*^{R121K} and *pho80*^{E154V}) that labilize the interaction between Pho80 and Pho81 and render it P_i-sensitive (45). We introduced these labilizing Pho80 substitutions into our inositol pyrophosphate mutants, which in addition expressed a fluorescent Pho81^{yEGFP}. Under P_i-replete conditions, wild-type cells expressing the *pho80*^{R121K} or *pho80*^{E154V} alleles as the sole source of Pho80 showed Pho81^{yEGFP} in the cytosol (Fig. 6). They accumulated the protein in the nucleus only upon P_i starvation. This accumulation was more pronounced in *pho80*^{R121K} than in *pho80*^{E154V}, suggesting that *pho80*^{E154V} affects the interaction with Pho81 more severely than *pho80*^{R121K}. Pho81$^{K154A\text{-}yEGFP}$, with a substitution inactivating its PP-IP binding site, accumulated in the nucleus of *pho80*^{R121K} and *pho80*^{E154V} cells already under P_i replete conditions, i.e., it behaved constitutively as under P_i starvation. *vip1Δ* mutants behaved like the wildtype, accumulating Pho81^{yEGFP} in the nucleus *of pho80*^{R121K} and *pho80*^{E154V} cells upon P_i starvation. These observations favor a model in which binding of inositol pyrophosphates to the SPX domain of Pho81 decreases the affinity between Pho81 and Pho80 and thereby relieves the inhibition of the Pho80/85 complex by Pho81. The SPX domain itself may contribute to the interaction with Pho80 because Pho81$^{\Delta SPX\text{-}yEGFP}$, which lacks this SPX domain, could not even accumulate in nuclei upon P_i starvation (Fig. 6). An interesting side-observation is that P_i-starved cells showed Pho81^{yEGFP} not only in the nucleus but also in small speckles which line the plasma membrane (Fig. 6). In pho80^{E154V} cells, they were already visible under P_i-replete conditions, suggesting that binding to nuclear Pho80 competes with the recruitment of Pho81 into peripheral speckles. The nature of these speckles is unknown and was not pursued further in this study.

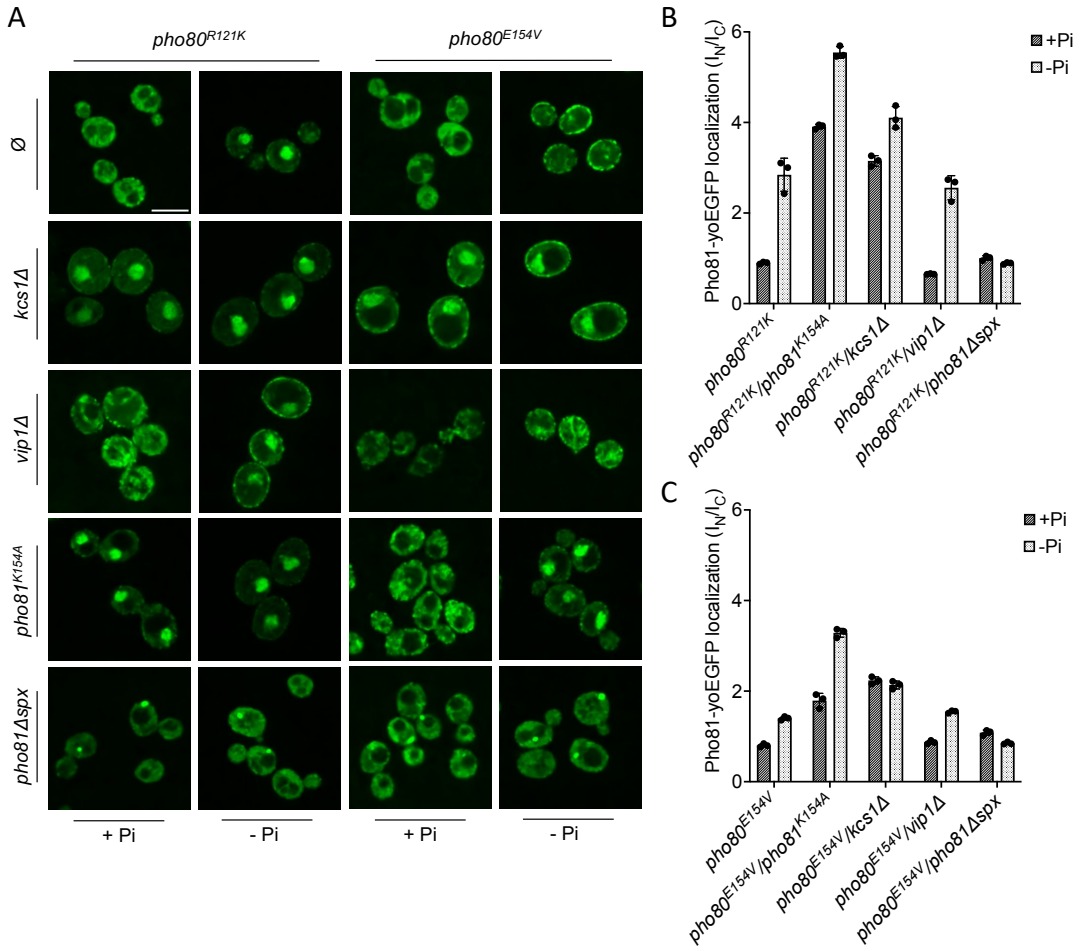


Fig. 6. Localization of Pho81^{yEGFP} in *pho80^{R121K}* and *pho80^{E154V}* loss-of-affinity mutants.

Cells were logarithmically grown in P_i-replete SC medium as in Fig. 2A, washed, and incubated for further 4 h in medium with 7.5 mM of P_i (+ P_i), or in starvation medium (- P_i).

(A) Pho81 imaging. Cells expressing Pho81^{yEGFP} and the indicated variants of Pho80 from their genomic loci were imaged on a spinning disc microscope after 4h of growth in the presence of 7.5 mM Pi (+ P_i), or after 4h of P_i starvation (- P_i). Scale bar = 5 µM. λ_{ex}: 488 nm; λ_{em}: 500-530 nm. Note that the fluorescent dots visible in *pho81$^{\Delta spx}$* cells are not nuclei because they are too small and at positions where nuclei are not found. Their location was not further investigated because it is not essential for this study.

(B) Quantification of the nuclear localization of Pho81^{yEGFP}in *pho80^{R121K}* cells. Images from A were subjected to automated segmentation and quantification of the average fluorescence intensity in the nucleus and cytosol as in Fig. 3B. 100-200 cells were quantified per sample. n=3 experiments.

(C) Quantification of the nuclear localization of Pho81^{yEGFP} in *pho80^{E154V}* mutants was performed as in B.

Discussion

The transcriptional response of *S. cerevisiae* to varying P_i supply is an important model system for studying gene regulation, promotor structure, and the impact of chromatin structure (38, 71). However, identification of the inositol pyrophosphates relaying phosphate availability to this PHO pathway has been challenging. This is mainly due to limitations in analytics. The recent development of a CE-MS method has provided a potent tool, allowing PP-IP analysis without radiolabeling and under any physiological condition. The improved separation, higher throughput and lower cost of this approach led us to re-investigate the role of PP-IPs in PHO pathway regulation. The results inspire a revised model differing from the traditional model in three aspects: We propose that P_i scarcity is signaled by a decrease in PP-IPs rather than by an increase; a critical function is ascribed to the loss of 1,5-IP_8 instead of 1-IP_7; and we postulate that the N-terminal SPX domain of Pho81 rather than the central minimum domain is the primary receptor mediating PP-IP-control over the PHO pathway.

Our observations reveal an effect that misled the interpretation of several preceding studies. This concerns our own work on the activation of the polyphosphate polymerase VTC. Our in vitro activity assays had shown a clear order of potency of PP-IPs in stimulating VTC, with 1,5-IP_8 showing an apparent EC_{50} 30-fold below that of 5-IP_7 and 50-fold below that of 1-IP_7 (4). In this work, we nevertheless dismissed 1,5-IP_8 as a physiological stimulator of the VTC SPX domains based on the argument that a *vip1Δ* mutant, which lacks the enzyme for 1,5-IP_8 synthesis, shows quite normal polyP accumulation in vivo. We proposed 5-IP_7 for this role because a *kcs1Δ* mutant, which lacks the IP6K making this compound, also lacks polyP. The PP-IP analyses that we present now call for a re-interpretation of this model. The presence of polyP in a *vip1Δ* mutant can well be explained by the 20-fold over-accumulation of 5-IP_7 in this mutant, which can compensate for the lower EC_{50} and permit stimulation of VTC through 5-IP_7. In wildtype cells, however, where 5-IP_7 is only twice as abundant as 1,5-IP_8 (Fig. 2), 1,5-IP_8 should be the relevant activator of VTC for polyP synthesis when P_i is abundant. Then, the situation is also compatible with results from S. pombe, where ablation of the Vip1 homolog Asp1 impairs polyP synthesis (20).

The transcriptional phosphate starvation response in S. pombe depends also on PP-IPs. However, the downstream executers differ from those present in S. cerevisiae. Fission yeast contains no homologs of Pho81, and the responsible transcription factor is not controlled

through a Pho80/Pho85-like kinase (28–30). Nevertheless, important basic properties of the IP6K/PPIP5K system are conserved. Like in baker's yeast, deletion of the Vip1 homolog Asp1 leads to a strong increase in IP_7 (23). Characterization of the purified enzyme showed that P_i stimulates net production of IP_8 by inhibiting the phosphatase domain of Asp1 (27). Thus, we must expect a decreased production of IP_8 upon P_i starvation in vivo also in S. pombe. As for Vip1 in baker's yeast, genetic ablation of Asp1 does not interfere with the induction of the phosphate-regulated gene PHO1 by P_i starvation, suggesting that 1-IP_7 and 1,5-IP_8 are not essential for this transcriptional response (28). In P_i-replete media, however, ablation of Asp1 hyper-represses PHO1, and putative overproduction of 1-IP_7 and/or 1,5-IP_8 by inactivation of the Asp1 phosphatase domain induces this gene (28, 72). It appears likely that this discrepancy also originates from a similar disequlibration of IP_7 species as in Saccharomyces because ablation of Asp1 increases the IP_7 pool 4- to 5-fold (23). This can be judged better once the changes of the different IP_7 isomers will have been dissected under different P_i regimes in these mutants.

The PHO pathway in Cryptococcus neoformans, a pathogenic yeast, shares many protein components with that of S. cerevisiae and might hence be expected to function in a similar manner (73). Yet a recent study on this organism proposed 5-IP_7 as the critical inducer of the PHO pathway (17). This model relies on the observation that *kcs1Δ* mutants, which lack 5-IP_7, cannot induce the PHO pathway in this yeast. This is the opposite behavior compared to S. cerevisiae. It was hence concluded that, despite an experimentally observed moderate decline in 5-IP_7 upon short P_i starvation, the remaining pool of 5-IP_7 was required for PHO pathway activity. Substitution of basic residues in the PP-IP-binding pocket of C. neoformans Pho81 interfered with PHO pathway activation, lending further support to the conclusion. However, the same study also showed that Pho81 was lacking completely from kcs1Δ mutants. When the Pho81 PP-IP binding site was compromised by amino acid substitutions the Pho81 protein level was significantly reduced, and the protein almost vanished upon P_i starvation. This offers an alternative explanation of the effects because absence of the critical inhibitor Pho81 activates Pho85/80 kinase and represses the PHO pathway independently of P_i availability (39, 40). Destabilization of the Pho81 will then mask effects of PP-IPs. Thus, it remains possible that the PHO pathway in C. neoformans is controlled as in S. cerevisiae, i.e. that P_i starvation is signaled by a decline of 1,5-IP_8. We analyzed the inositol pyrophosphates of C. neoformans

by CE-MS. Upon shifting the cells from P_i-replete to P_i-starvation medium, all inositol pyrophosphates disappeared within 2 hours (Fig. S5). As in S. cerevisiae, 1,5-IP_8 showed a faster and more profound response, becoming undetectable already after 20 min of P_i-starvation. Therefore, we consider it as likely that the loss of 1,5-IP_8 signals P_i starvation also in C. neoformans.

Our results revise the widely accepted model of increased 1-IP_7 as the trigger of the PHO pathway and the minimum domain of Pho81 as its receptor (15, 16). This model rests upon the increase of 1-IP_7 upon P_i starvation, a reported constitutive activation of the PHO pathway in a *ddp1Δ* mutant, and upon the lack of rapid PHO pathway induction in a *vip1Δ* mutant. A later study from this group revealed that *vip1Δ* cells finally induced the PHO pathway after prolonged P_i starvation (74), but this did not lead to a revision of the model. Our results rationalize the strong delay of *vip1Δ* in PHO pathway induction by the overaccumulation of 5-IP_7 in this mutant, which leads to aberrant PHO pathway silencing through this compound. Under P_i starvation, it takes several hours longer to degrade this exaggerated pool, which provides a satisfying explanation for the delayed PHO pathway induction in *vip1Δ* cells. Even though earlier studies of O'Shea and colleagues, due to the limited resolution of the radio-HPLC approach, could not differentiate between 1- and 5-IP_7, these studies reported an increase in the IP_7 pool upon P_i starvation (15). Other studies using a similar radio-HPLC approach suggested a decline of the IP_7 pool upon long P_i withdrawal, but they analyzed only a single timepoint and could not exclude an earlier increase in 1-IP_7 as a trigger for the PHO pathway (6, 17, 18). In our CE-MS analyses, we consistently observed strong, continuous declines of 1,5-IP_8, 5-IP_7 and 1-IP_7 and, upon several hours of P_i starvation, their almost complete disappearance. Furthermore, we could not confirm the reported constitutive activation of the PHO pathway in a *ddp1Δ* mutant (15), although this mutant did show the expected increased 1-IP7 concentration. These observations argue against a role of 1-IP_7, 5-IP_7 or 1,5-IP_8 as activators for Pho81 and the PHO pathway.

The link between 1-IP_7 and Pho81 activation relied mainly on the use of the 80 amino acid minimum domain from the central region (15, 16, 45). This domain was used in *in vitro* experiments, where 1-IP_7 stimulated binding of the minimum domain to Pho80, thereby inhibiting Pho85/Pho80. Given that 1-IP_7 disappears under P_i starvation it appears unlikely

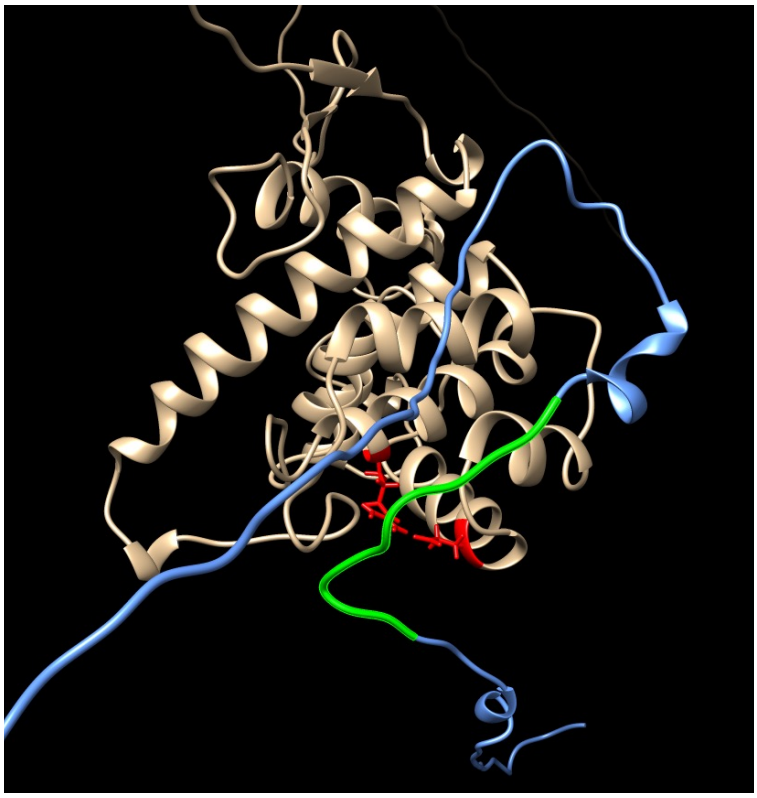
that this in vitro effect is physiologically relevant. Furthermore, the apparent dissociation constant for the complex of 1-IP_7 with the minimum domain and Pho80/Pho85 was determined to be 20 µM (16). The minimum domain is thus unlikely to respond to 1-IP_7 in the cells because its K_d is almost 50-fold above the cellular concentrations of 1-IP_7 in P_i-replete medium (0.5 µM; Fig. 2), and at least 3 orders of magnitude above the concentration remaining on P_i starvation media (< 20 nM). Another discrepancy is that the *in vitro* inhibition of Pho80/Pho85 by the minimum domain was reported to be specific for 1-IP_7 whereas 5-IP_7 had no effect (16). This contrasts with the in vivo situation, where we find that even massive overaccumulation of 1-IP_7 by ablation of Ddp1 cannot trigger the PHO pathway, but where suppression of 5-IP_7 and 1,5-IP_8 production by ablation of Kcs1 does activate it constitutively (Fig. 3)(19).

An argument supporting a role of the minimum domain in mediating P_i-responsiveness was provided by in vivo experiments in a pho81Δ mutant, where P_i-dependent activation of the PHO pathway could be partially rescued through over-expression of the minimum domain (40, 45). PHO pathway activation was assayed through PHO5 expression. This is relevant because the PHO5 promotor is not only controlled through Pho85/Pho80-mediated phosphorylation of Pho4, but also through changes in nucleosome occupancy, which substantially influence activity and activation threshold of the PHO5 promotor (67, 69). It is thus conceivable that constitutive overexpression of the minimum domain reduces Pho85/Pho80 activity moderately, but sufficiently to allow regulation of PHO5 through nucleosome positioning, which changes in a P_i-dependent manner (67).

Even though the minimum domain is unlikely to function as a receptor for PP-IPs this does not draw into question that the minimum domain is critical for Pho81 function. This is suggested by substitutions and deletions in this domain, introduced into full-length Pho81, which constitutively repress the PHO pathway (40, 45). We hence explored the effects of the minimum domain by molecular modeling. Using Alphafold Multimer we generated models of Pho80 in complex with the minimum domain (Fig. 7). The structure predictions place the minimum domain in a groove of Pho80. This groove contains both E154 and R121, the two residues that were identified by a random mutagenesis approach as critical for the interaction between Pho80 and Pho81 (45). Modeling a Pho81/Pho85/Pho80 complex yielded a similar

result. Also here, the minimum domain is modeled as a long unstructured loop associated with the groove containing E154 and R121. In this model, the minimum domain provides the contact between Pho80 and Pho81. This readily explains why substitutions in the Pho81 minimum domain (residues 690-701) as well as in Pho80 residues E154 or R121 destabilize the association of Pho81 with Pho85/Pho80 (75). The effects of substituting these residues lend credibility to this aspect of the structure prediction.

A



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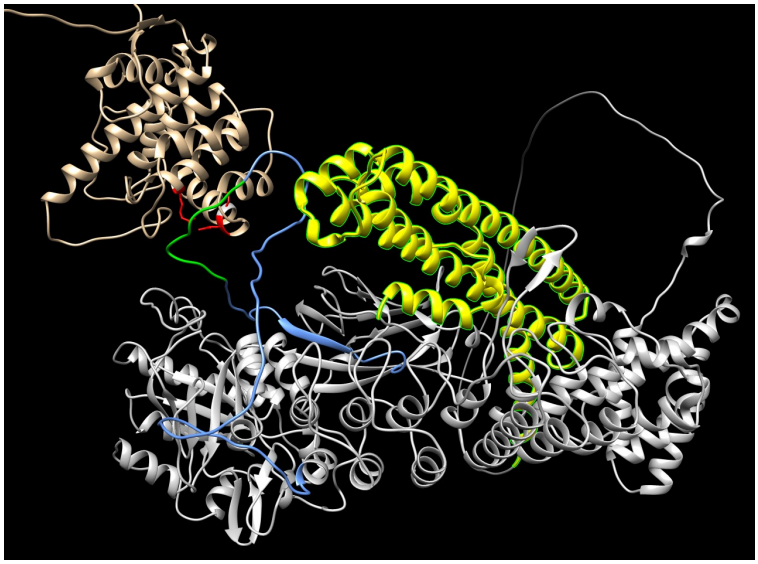

Fig. 7: Structure predictions of the minimum domain in complex with Pho80
Alphafold multimer was used to generate the following structure predictions:
(A) Pho80 in complex with the minimum domain of Pho81 (aa 645-724).
(B) Pho80 in complex with Pho81.
Coloring: Beige - Pho80; red - E154, R121 of Pho80; blue: Pho81 minimum domain; green - central region of the Pho81 minimum domain that is critical for Pho85/Pho80 inhibition (aa 690-701); yellow: SPX domain of Pho81; grey - rest of Pho81 without assigned function.

If the minimum domain itself may not serve as PP-IP receptor of Pho81 but is important for regulating Pho80/Pho85 activity, how does Pho81 receive information about cytosolic P_i levels? Our in vivo analyses of Pho81 regulation were performed with the full-length protein. In this context, the SPX domain exerts strong control over Pho81 activity, because a single substitution of one of the lysines in the PP-IP-binding patch, designed to mimic the PP-IP-free state (6), sufficed to induce Pho4 translocation and PHO pathway activation. This critical role of the SPX domain for controlling Pho81 is consistent with the results from two random mutagenesis approaches (40, 48), which had received little attention in the previous model of PHO pathway regulation (15, 16). These random mutagenesis approaches generated multiple pho81^c alleles, which constitutively activate the PHO pathway (G4D, G147R, 158-SGSG-159, E79K). All these alleles affect the SPX domain at residues in the immediate vicinity of the PP-IP binding site (Fig. S3). Three of the alleles (G4D, G147R, 158-SGSG-159) replace small glycine residues by bulky residues or insert additional amino acids, respectively, at sites where they should sterically interfere with binding of inositol pyrophosphates (Fig. 8). We hence expect them to mimic the PP-IP-free state. In sum, multiple lines of evidence support the view that the SPX domain exerts dominant, 1,5-IP_8 mediated control over Pho81 activity in response to P_i availability.

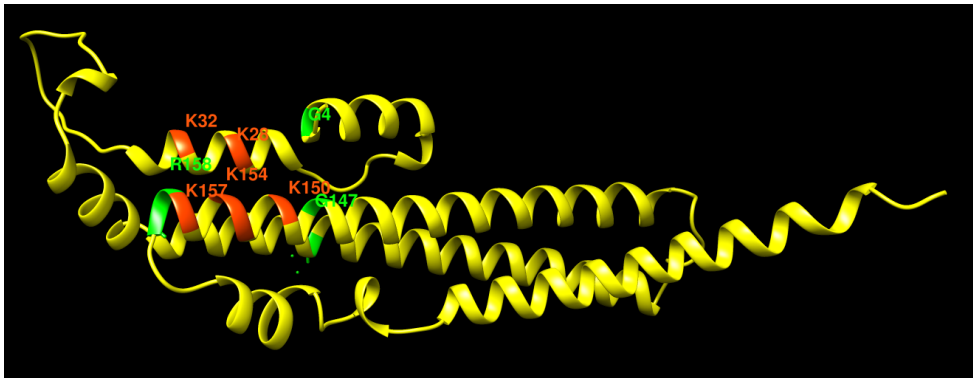

Fig. 8: Pho81 residues leading to constitutive activation of the PHO pathway
The image shows an Alphafold prediction of the Pho81 SPX domain (aa 1-215) in yellow. Basic residues of the PP-IP binding site have been identified by structure matching with the IP_6-associated SPX domain of VTC4 from Chaetomium thermophilum (PDB 5IJP). They are labeled in red. Residues from random mutagenesis screens, which lead to constitutive activation of the PHO pathway are labeled in green.

Taking all these aspects together, we propose the following working model (Fig. 9): P_i sufficiency in the cytosol is signaled through the accumulation of 1,5-IP_8. Its binding to the SPX domain of Pho81 inactivates this CDK inhibitor and labilizes its interaction with Pho85/Pho80. The now un-inhibited kinase phosphorylates Pho4, triggers its relocation into the cytosol and represses the PHO pathway. P_i limitation is signaled by a decline in 1,5-IP_8, resulting in the ligand-free form of the SPX domain and the activation of Pho81. Activation of Pho81 allows its minimum domain to bind a groove in the cyclin Pho80, which stabilizes the complex and inhibits Pho85 kinase. An obvious question arising from this model is how the availability of the minimum domain for Pho80 binding could be controlled. An attractive speculation is that this control could be exerted through an interaction with the SPX domain. This aspect will be explored in future work.

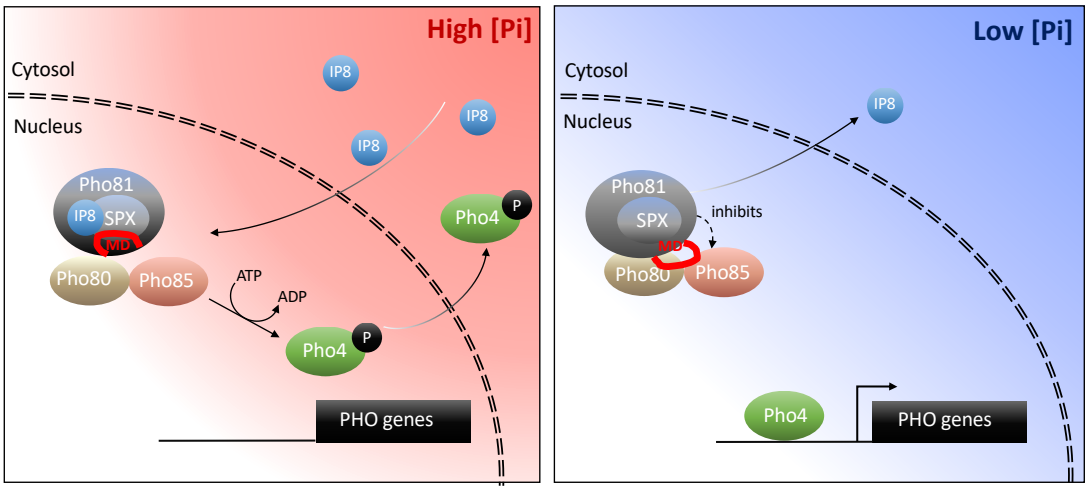

Fig. 9: Working model on the control of the PHO pathway through 1,5-IP_8 and Pho81.
At high P_i concentrations, inositol pyrophosphates accumulate. 1,5-IP_8 preferentially binds the SPX domain of Pho81, which labilizes the interaction of Pho81 with Pho80 and prevents Pho81 from inhibiting Pho85/Pho80 kinase.

In low P_i, 1,5-IP_8 and other pyrophosphates decline. Liberation of the SPX domain from this ligand allows Pho81 to insert its minimum domain into a critical cleft of Pho80, binding Pho80-85 more tightly and inhibiting it. This allows dephosphorylation of Pho4, its concentration in the nucleus and activation of the PHO pathway. This inhibition is reinforced by increased expression of PHO81, which itself is a PHO pathway-controlled gene.

This revision of PHO pathway regulation permits to develop a coherent picture on intracellular phosphate signaling across the eukaryotic kingdoms. Phosphate abundance being indicated by the accumulation of 1,5-IP_8 is compatible with the situation in mammalian cells, where 1,5-IP_8 triggers P_i export through the putative phosphate transporter XPR1 (11, 76), and with the situation in plants where IP_8 are only abundant on high-P_i substrate and suppresses the phosphate starvation response (10). Thus, we propose that phosphate scarcity in eukaryotes is generally signaled through a loss of 1,5-IP_8.

Materials and Methods

S. cerevisiae culture

Cells have been grown in liquid Synthetic Complete (SC) medium of the following composition: 3.25 g YNB without amino acids (Difco 291920); 1 g Synthetic Complete Mixture (Kaiser) Drop-Out: Complete (Formedium DSCK1000); 50 ml 20% glucose; 450 ml demineralized water. Phosphate starvation experiments were performed in SC-P_i medium: 3.25g YNB without amino acids and phosphate, supplemented with KCl (Formedium CYN6703); 1 g Synthetic Complete Mixture (Kaiser) Drop-Out: Complete (Formedium DSCK1000); 50ml 20% glucose; 450 ml water. These synthetic media were sterile filtered and tested for absence of free P_i by the malachite green assay. Cultures were shaken in capped Erlenmeyer flasks at 150 rpm at 30°C unless stated otherwise.

Yeast strains and their genetic manipulation

The *S. cerevisiae* strains used in this study are listed and described in the Electronic Supporting Information. DNA transformations were carried out with overnight cultures at 30°C in YPD medium (Yeast Extract-Peptone-Dextrose) using the following protocol: Cultures (not exceeding 10^7 cells/mL) were centrifuged at 1800 x g for 2 min. 25 µL of pellet was resuspended in 25 µL of LiAc-TE 0.1 M (Lithium acetate 0.1M, Tris 10 mM, EDTA 1 mM). The cell suspension was supplemented with 240 µL of PEG 50%, 36 µL of LiAC 1 M, 5 µL of boiled ssDNA, and the DNA (200 ng of plasmid and/or 0.5-2 mg of PCR product). After 30 min at 30°C, a heat shock was carried out at 42°C for 20 min. Cells were then centrifuged at 1800 x g for 2 min, resuspended in 150 µL of water, and grown on selective plates for 2 to 4 days. Fluorescent protein tags have been obtained using a standard method (77), except for Hta2 tagging, which was performed by CRISPR-Cas9. All introduced single mutations, knockouts, and fluorescent protein tags were confirmed by PCR and sequencing.

Plasmid constructions

The plasmids used to carry out CRISPR-Cas9 genome editing were obtained by cloning hybridized oligonucleotides coding for the sgRNA between the XbaI and AatII restriction sites of the parent plasmid (pSP473, pSP475 or pSP476). The plasmids used to overexpress Vip1 (pVC97 and pVC114) were obtained by cloning the *vip1* open reading frame between the BamHI and SalI restriction sites of the parent plasmid (respectively pRS415-GDP and pRS413-GPD) (78). The plasmid used to monitor prPho5-GFP expression (pVC115) was obtained by replacing the *pgk1* promoter with the -1 to -1500 region of the *pho5* promoter and by replacing the gene for G418 resistance to the coding region of the *leu2* gene within the pCEV-G1 backbone (Addgene).

Fluorescence microscopy

Cells were grown logarithmically in SC medium for 12-15 h until they reached a density of 10^7 cells/mL. For P_i starvation experiments, overnight-grown cells were washed twice, resuspended in SC-P_i medium at a density of ca. $5x10^6$ cells/mL, and incubated at 30°C. Fluorescence images were recorded on a Nikon Eclipse Ti2/Yokogawa CSU-X1 Spinning Disk microscope equipped with two Prime BSI sCMOS cameras (Teledyne Photometrics), a LightHUB Ultra® Laser Light (Omicron Laserage), and an Apo TIRF 100x/1.49 Oil lens (Nikon).
Time-lapse experiments were performed on a Nikon Eclipse Ti2 inverted microscope equipped with a Prime BSI sCMOS camera (Teledyne Photometrics), a Spectra X Light Engine (Lumencor), a Plan Apo λ 100x/1.45 Oil lens (Nikon). Microscopy chambers were made using sticky-Slides VI $^{0.4}$ (Ibidi) glued onto concanavalin a-coated cover slips (Knittel glass, 24 x 60 x 0.13 – 0.17 mm). The cell suspension (200 µL, 10^7 cells/mL) was added to the chamber. After 30 seconds of sedimentation, a flow of SC medium was applied using the microfluidic flow controller AF1 Mk2 (Elveflow) coupled to a MUX distributor (Elveflow). After 2 min, the flow was switched to SC-P_i medium, and imaging was started. After further 2 min, the flow was stopped, and the time lapse was continued by recording one image every 5 min. The temperature was kept at 30°C using the stage-top incubator Uno-Controller (Okolab).

Western Blots

5 mL of cells grown overnight in SC medium (until ca. 10^7 cells/mL) was centrifuged (1800 x g, 2 min). Cells were resuspended in 1 mL of lithium acetate (2 M) and centrifuged as before. Cells were resuspended in 1 mL of sodium hydroxide (0.4 M) and kept on ice for 5 min. After centrifugation (1800 x g, 2 min), cells were resuspended in 100 µL of SDS-PAGE Sample Buffer and boiled for 5 min. After centrifugation under the same conditions, the supernatant was collected, and the protein content was measured using a NanoDrop 1000 Spectrophotometer (Witec). Supernatant concentrations were adjusted, and the samples were loaded on SDS-polyacrylamide gels.

Inositol pyrophosphate quantification

Cells were logarithmically grown in SC medium overnight to reach a density of 10^7 cells/mL. 1 mL of cell culture was mixed with 100 µL of 11 M perchloric acid (Roth HN51.3). After snap-freezing in liquid nitrogen, the samples were thawed and cell debris was removed by centrifugation (15'000 x g, 3 min, 4°C). Titanium dioxide beads (Titansphere TiO beads 5 mm, GL Sciences 5020-75000) were pretreated by washing them once with water, once with 1 M PA and finally resuspending them in 200 µl 1 M PA. The cell supernatant was mixed with 1.5 mg of beads and incubated with shaking or on a rotating wheel for 15 min at 4°C. The beads were collected by centrifugation (15'000 x g, 1 min, 4°C) and washed twice with 1 M perchloric acid. Inositol phosphates were eluted by 300 mL of 3% ammonium hydroxide and the eluate was evaporated using a speed-vac concentrator at 42°C and 2000 rpm overnight. The pellet was resuspended using 20 ml of water. CE-ESI-MS analysis was performed on an Agilent 7100 CE coupled to triple quadrupole mass spectrometer (QqQ MS) Agilent 6495c, equipped with an Agilent Jet Stream (AJS) electrospray ionization (ESI) source. Stable CE-ESI-MS coupling was enabled by a commercial sheath liquid coaxial interface, with an isocratic LC pump constantly delivering the sheath-liquid.

Spectrofluorimetry

Cells were grown overnight to a density of $5x10^6$ cells/mL in SC medium. They were then resuspended in SC-P_i medium after two washing steps. For each timepoint, the concentration of the cell suspension was measured and adjusted to 10^7 cells/mL before fluorescence measurement. The fluorescence of yEGFP (λ_{ex}: 400 nm; λ_{em}: 500-600 nm; λ_{cutoff}: 495 nm) and mCherry (λ_{ex}: 580 nm; λ_{em}: 600-700 nm; λ_{cutoff}: 590 nm) were measured on a SpectraMax Gemini EM microplate fluorimeter at 30°C.

Analysis of IPP in C. neoformans

C. neoformans grubii wildtype cells were grown in liquid synthetic complete (SC) medium to logarithmic phase. They were sedimented (3000 x g, 1 min, 4°C), washed twice with phosphate-free medium, aliquoted, and used to inoculate four 25 ml cultures in SD medium lacking phosphate. The inoculum was adjusted (starting OD_{600} at 0.9, 0.6, 0.4, 0.3 etc, respectively) such that after further incubation for 0 to 240 min all samples had similar OD_{600} at the time of harvesting. The sample for the 0 min timepoint was taken from the culture in SC medium. The starvation time was counted beginning with the first wash. At the indicated time points, the OD_{600} of the culture was measured. A 4 ml sample was collected, supplemented with 400 µl of 11 M perchloric acid (PA), frozen in liquid nitrogen and kept at -80°C. The samples were thawed and IPPs were extracted using 6 mg TiO_2 beads due to the higher number of cells. All further steps were as described above for S. cerevisiae samples.

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Supplementary Information

Strains

The *S. cerevisiae* strains used in this study were all obtained by genetic manipulations from the BY4741 background described in Table S1. This strain corresponds to the wild-type of this study. For the sake of clarity, the BY4741 background was therefore not indicated in the genotype of each mutant strain.

Table S1. Strains used in this study

Strain	Genotype	Plasmid	Source
wild-type (BY4741)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*		Euroscarf
pho4-yoEGFP	*pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
pho81-yoEGFP	*pho81-link-yoEGFP-CaURA3*		This study
pho81Δspx	*pho81Δ200*[a]		This study
pho81Δspx/pho4-yoEGFP	*pho81Δ200*[a] *pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
pho81Δspx-yoEGFP	*pho81Δ200*[a] *pho81-link-yoEGFP-CaURA3*		This study
pho81Δspx/prPho5-yEGFP	*pho81Δ200*[a]	pCEV-G1	This study
vip1Δ	*vip1Δ0*[a]		This study
vip1Δ/pho4-yoEGFP	*vip1Δ0*[a] *pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
vip1Δ/pho81-yoEGFP	*vip1Δ0*[a] *pho81-link-yoEGFP-CaURA3*		This study
vip1Δ/prPho5-yEGFP	*vip1Δ0*[a]	pCEV-G1	This study
kcs1Δ	*kcs1Δ0::kanMX40*		[1]
kcs1Δ/pho4-yoEGFP	*kcs1Δ0::kanMX40 pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
kcs1Δ/pho81-yoEGFP	*kcs1Δ0::kanMX40 pho81-link-yoEGFP-CaURA3*		This study
kcs1Δ/prPho5-yEGFP	*kcs1Δ0::kanMX40*	pCEV-G1	This study
vip1Δ/kcs1Δ	*vip1Δ0*[a] *kcs1Δ0::kanMX40*		This study
vip1Δ/kcs1Δ/pho4-yoEGFP	*vip1Δ0*[a] *kcs1Δ0::kanMX40 pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
vip1Δ/kcs1Δ/pho81-yoEGFP	*vip1Δ0*[a] *kcs1Δ0::kanMX40 pho81-link-yoEGFP-CaURA3*		This study
vip1Δ/kcs1Δ/prPho5-yEGFP	*vip1Δ0*[a] *kcs1Δ0::kanMX40*	pCEV-G1	This study
prGPD-vip1/kcs1Δ	*kcs1Δ0::kanMX40*	pVC97	This study

prGPD-vip1/kcs1Δ/pho4-yoEGFP	*kcs1Δ0::kanMX40 pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]	pVC97	This study
prGPD-vip1/kcs1Δ/pho81-yoEGFP	*kcs1Δ0::kanMX40 pho81-link-yoEGFP-CaURA3*	pVC97	This study
prGPD-vip1/kcs1Δ/prPho5-yEGFP	*kcs1Δ0::kanMX40*	pCEV-G1, pVC114	This study
pho81^{K154A}	*pho81*^{K154A} [a]		This study
pho81^{K154A}*/pho4-yoEGFP*	*pho81*^{K154A} [a] *pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
pho81^{K154A}*/pho81-yoEGFP*	*pho81*^{K154A}*-link-yoEGFP-CaURA3*[a]		This study
pho81^{K154A}*/prPho5-yEGFP*	*pho81*^{K154A} [a]	pCEV-G1	This study
pho81^{K154A}*/vip1Δ/pho4-yoEGFP*	*pho81*^{K154A} [a] *vip1Δ0*[a] *pho4-link-yoEGFP-CaURA3 hta2-mCherry*[a]		This study
pho80^{R121K}*/pho81-yoEGFP*	*pho81*^{R121K} [a] *pho81-link-yoEGFP-CaURA3*		This study
pho80^{R121K}*/kcs1Δ/pho81-yoEGFP*	*pho81*^{R121K} [a] *kcs1Δ0::kanMX40 pho81-link-yoEGFP-CaURA3*		This study
pho80^{R121K}*/vip1Δ/pho81-yoEGFP*	*pho81*^{R121K} [a] *vip1Δ0*[a] *pho81-link-yoEGFP-CaURA3*		This study
pho80^{R121K}*/pho81*^{K154A}*-yoEGFP*	*pho81*^{R121K} [a] *pho81*^{K154A}*-link-yoEGFP-CaURA3*[a]		This study
pho80^{R121K}*/pho81Δspx-yoEGFP*	*pho81*^{R121K} [a] *pho81Δ200-link-yoEGFP-CaURA3*[a]		This study
pho80^{E154V}*/pho81-yoEGFP*	*pho80*^{E154V} [a] *pho81-link-yoEGFP-CaURA3*		This study
pho80^{E154V}*/kcs1Δ/pho81-yoEGFP*	*pho80*^{E154V} [a] *kcs1Δ0::kanMX40 pho81-link-yoEGFP-CaURA3*		This study
pho80^{E154V}*/vip1Δ/pho81-yoEGFP*	*pho80*^{E154V} [a] *vip1Δ0*[a] *pho81-link-yoEGFP-CaURA3*		This study
pho80^{E154V}*/pho81*^{K154A}*-yoEGFP*	*pho80*^{E154V} [a] *pho81*^{K154A}*-link-yoEGFP-CaURA3*[a]		This study
pho80^{E154V}*/pho81Δspx-yoEGFP*	*pho80*^{E154V} [a] *pho81Δ200-link-yoEGFP-CaURA3*[a]		This study

[a] obtained by the CRISPR-Cas9 method

Plasmids

Table S2. Plasmids used in this study

Plasmid	Description	Source
pVC115	pRS425 *prPHO5-yegfp prTEF-mcherry leu2*	This study
pVC97	pRS415 *prGPD-vip1 leu2*	This study
pVC114	pRS413 *prGPD-vip1 his3*	This study
pED109	*mcherry*	Serge Pelet
pSP473	pRS423 *prGPD-cas9 his3*	Serge Pelet
pSP475	pRS425 *prGPD-cas9 leu2*	Serge Pelet
pSP476	pRS426 *prGPD-cas9 ura3*	Serge Pelet
pSP478	pRS425 *prGPD-cas9_sgRNA_hta2 leu2*	Serge Pelet
pVC24	pRS425 *prGPD-cas9_sgRNA_pho81 leu2*	This study
pVC43	pRS425 *prGPD-cas9_sgRNA_kcs1 leu2*	This study
pVC44	pRS423 *prGPD-cas9_sgRNA_vip1 his3*	This study
pVC110	pRS425 *prGPD-cas9_sgRNA_vip1 leu2*	This study
pVC72	pRS423 *prGPD-cas9_sgRNA_pho80_1 his3*	This study
pVC73	pRS423 *prGPD-cas9_sgRNA_pho80_2 his3*	This study
pVC108	pRS425 *prGPD-cas9_sgRNA_pho80_1 leu2*	This study
pVC109	pRS425 *prGPD-cas9_sgRNA_pho80_2 leu2*	This study
pVC97	pRS415 *prGPD-vip1 leu2*	This study
pVC114	pRS413 *prGPD-vip1 his3*	This study

Oligonucleotides

Table S3. Oligonucleotides used in this study

Oligonucleotide	Sequence
645_fw	acttgttgccaaagaagtctgccaagactgccaaagcttctcaagaactggcggccgctctagaacta
1435_rev	tactatacactgtctttaataaaacgacgctatttataaattatttagaagtggcgcgcc
CRISPR_sg_F	ggtcaaacgctgtagaagtg
Pho81_K150A_F2	tatgttgagttaaataaaacgggattttcagcagctctgaagaaatgggacaagagatctcaatctcacgataaagatttttatcttgctactgttgtttccattcaa ccaatt
Pho81_K150A_R2	aattggttgaatggaaacaacagtagcaagataaaaatctttatcgtgagattgagatctcttgtcccatttcttcagagctgctgaaaatcccgttttatttaactc aacata
Pho81_K154A_F2	aataaaacgggattttcaaaagctctgaaggcatgggacaagagatctcaatctcacgataaagatttttatcttgctactgttgtttccattcaaccaatt
Pho81_K154A_R2	aattggttgaatggaaacaacagtagcaagataaaaatctttatcgtgagattgagatctcttgtcccatgccttcagagcttttgaaaatcccgttttatt
Pho81_sgRNA_F	ttcaaaagctctgaagaaatgttttagag
Pho81_sgRNA_R	ctagctctaaaacatttcttcagagcttttgaaacgt
Vip1_sgRNA_F	tcgtagcatattaatatattgcagaaggtctgatcacacccaatttttaatttagtaacc
Vip1_sgRNA_R	ggttactaaattaaaaattgggtgtgatcagaccttctgcaatatattaatatgctacga
Kcs1_sgRNA_F	ttgtatatataaaactaaagctaaaagactaaagaaaggatagaactaatgaatattctt
Kcs1_sgRNA_R	aagaatattcattagttctatcctttctttagtcttttagctttagttttatatatacaa
Pho80_sgRNA_F1	ttaattcgttgactgcccatgttttagag
Pho80_sgRNA_R1	ctagctctaaaacatgggcagtcaacgaattaaacgt
Pho80_sgRNA_F2	atgtcacgaattgaatatacgttttagag
Pho80_sgRNA_R2	ctagctctaaaacgtatattcaattcgtgacatacgt
Pho80_R121K_F	attttacgcttaattcgttgactgcccataaatttttattaacagccaccacagtcgcaa
Pho80_R121K_R	ttgcgactgtggtggctgttaataaaaatttatgggcagtcaacgaattaagcgtaaaat
Pho80_E154V_F	atgcaaaagttggaggagtacgatgtcacgttttgaatatacttgagaacgatttttttaaagagagtaaactac
Pho80_E154V_R	gtagtttactctctttaaaaaatcgttctcaagtatattcaaaacgtgacatcgtactcctccaacttttgcat

Pho81_yoEGFP_F	ctacgcttgtgagttgctttttgagaataatattgatatgggtgacggtgctggttta
Pho81_yoEGFP_R	taatgtataagatttcaaaactacatattacagaactttatcgatgaattcgagctcg
Pho81Δspx_F	gaggcaacatagatagataaacgtgcaatggatattgacaacaacaatagaagggcagac
Pho81Δspx_R	gtctgcccttctattgttgttgtcaatatccattgcacgtttatctatctatgttgcctc
Vip1_F	ggggatccatgagtgggataaagaaggaacc
Vip1_R	cccgtcgacctaatctaatgtcttgttaacgg
Vip1_F2	aataacctcaccggtcacgg
Vip1_R2	gtaagacgttatttcccacc

CE-ESI-MS parameters

All experiments were performed with a bare fused silica capillary with a length of 100 cm and 50 µm internal diameter. 35 mM ammonium acetate titrated by ammonia solution to pH 9.7 was used as background electrolyte (BGE). The sheath liquid was composed of a water-isopropanol (1:1) mixture, with a flow rate of 10 µL/min. 15 µL Inositol pyrophosphate extracts was spiked with 0.75 µL isotopic internal standards mixture (2 µM $[^{13}C_6]1IP_8$, 10 µM $[^{13}C_6]5$-IP_7, 10 µM $[^{13}C_6]1$-IP_7 and 40 µM $[^{13}C_6]IP_6$). Samples were introduced by applying 100 mbar pressure for 10 s (20 nL). A separation voltage of +30 kV was applied over the capillary, generating a constant CE current at around 19 µA.

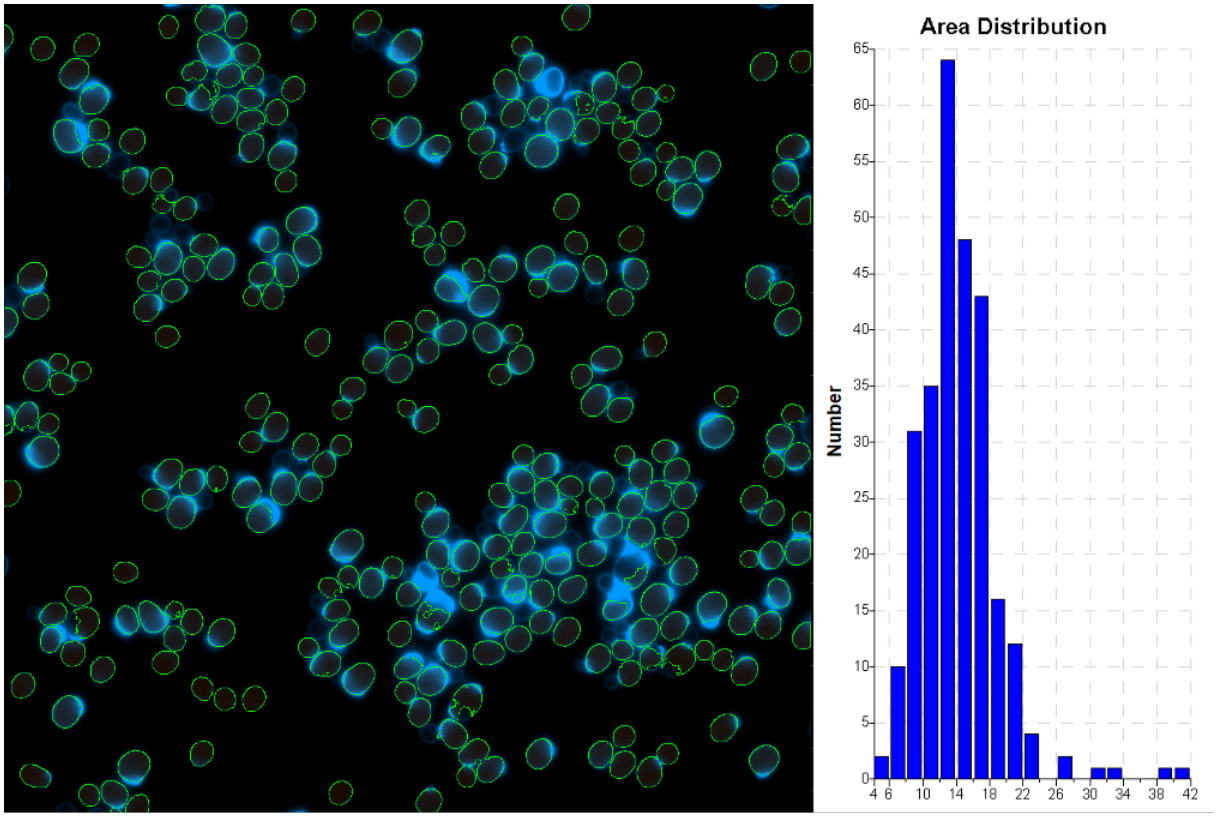
The MS source parameters setting were as follows: nebulizer pressure was 8 psi, sheath gas temperature was 175 °C and with a flow at 8 L/min, gas temperature was 150 °C and, with a flow of 11 L/min, the capillary voltage was -2000 V with nozzle voltage 2000V. Negative high pressure RF and low pressure RF (Ion Funnel parameters) was 70V and 40V, respectively. Parameters for MRM transitions are in Table S4.

Table S4. Parameters for MRM transitions

Compound	Precursor Ion	Product Ion	dwell	CE (V)	Cell Acc (V)	Polarity
$[^{13}C_6]IP_8$	411.9	362.9	80	10	1	Negative

IP_8	408.9	359.9	80	10	1	Negative
$[^{13}C_6]IP_7$	371.9	322.9	80	10	1	Negative
IP_7	368.9	319.9	80	10	1	Negative
$[^{13}C_6]IP_6$	331.9	486.9	80	17	1	Negative
IP_6	328.9	480.9	80	17	1	Negative

Supplementary Figures



Feature	Mean	St.Dev	Minimum	Maximum
Area [µm²]	14.26	4.57	5.96	40.8
EqDiameter [µm]	4.21	0.64	2.75	7.21
VolumeEqSphere [µm³]	41.99	21.84	10.94	196.02

Fig. S1. Determination of cell dimensions from trypan blue stained wild-type cells.

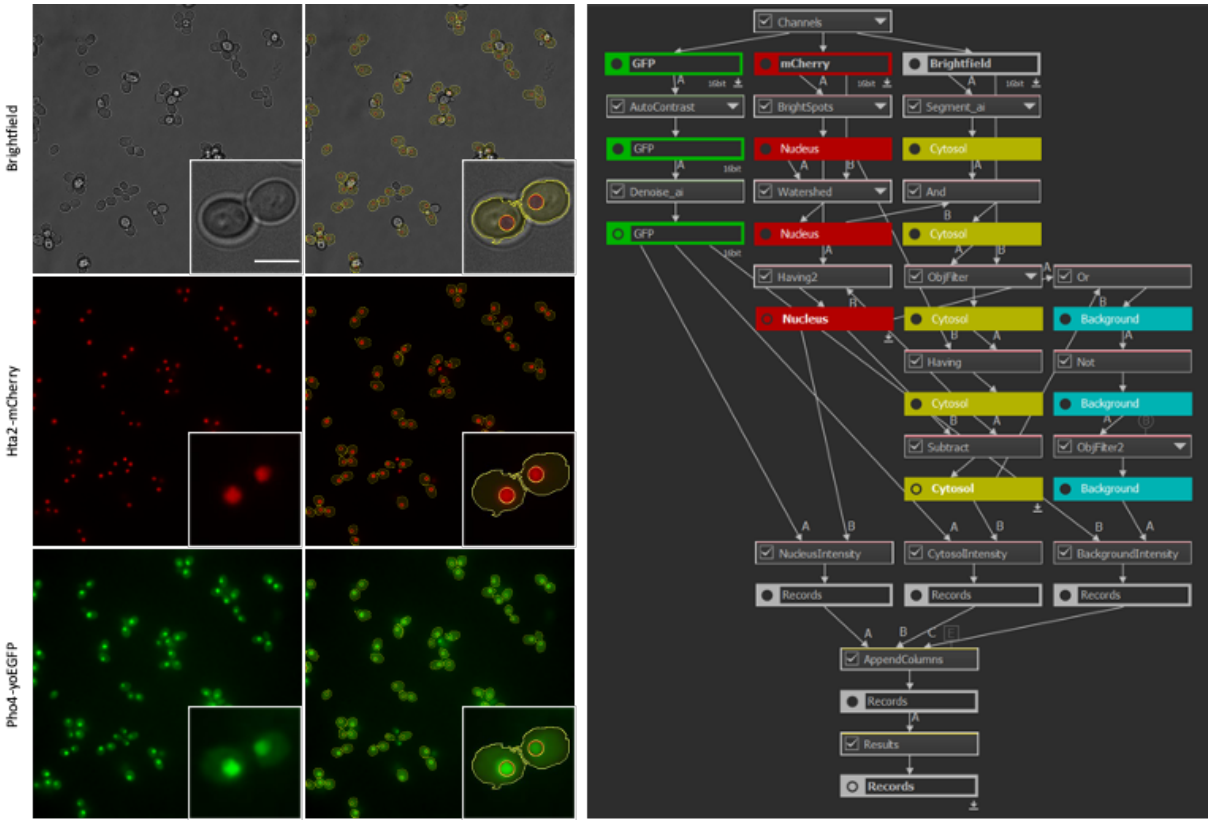


Fig. S2. Segmentation of fluorescence microscopy time-lapse experiments.
The subcellular localization of Pho4-yoEGFP has been quantified from time-lapse fluorescence microscopy experiments. This quantification required the segmentation of microscopy images to discriminate cytosolic and nuclear compartments. To this end, the hta2 histone of Pho4-yoEGFP strains has been tagged with mCherry. Nuclei were segmented using mCherry fluorescence images while the cell contours were segmented using brightfield images. Cell segmentation and the fluorescence quantification were performed using the General Analysis 3 module of the NIS Elements software (Nikon). (A) The brightfield and two fluorescence images of the same field are shown. Yellow lines indicate the boundaries of the cells and nuclei that have been recognized by the algorithm. (B) Flow chart of the commands of the NIS Elements General Analysis 3 suite used for the segmentation. The script has been deposited and can be downloaded from ******.

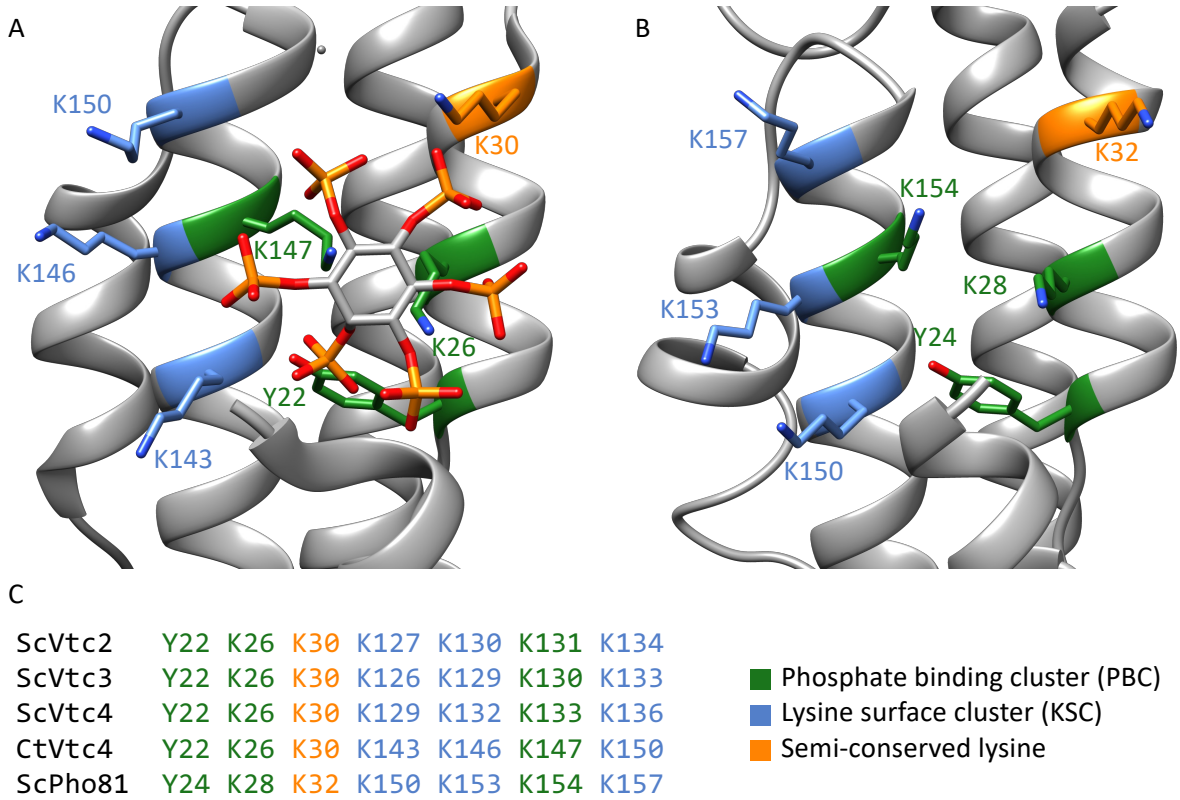


Fig. S3. *Myo*-inositol polyphosphate binding pocket of yeast SPX domains. (A) Crystal structure close-up view of *C. thermophilum* Vtc4 SPX domain complexing IP6 (5IJP). (B) Structure prediction (generated with SWISS-MODEL) of the *S. cerevisiae* Pho81 SPX domain revealing the potential conserved PBC and KSC clusters. (C) Alignment of the conserved residues *myo*-inositol polyphosphate binding motifs of Pho81 and of the different VTC subunits.

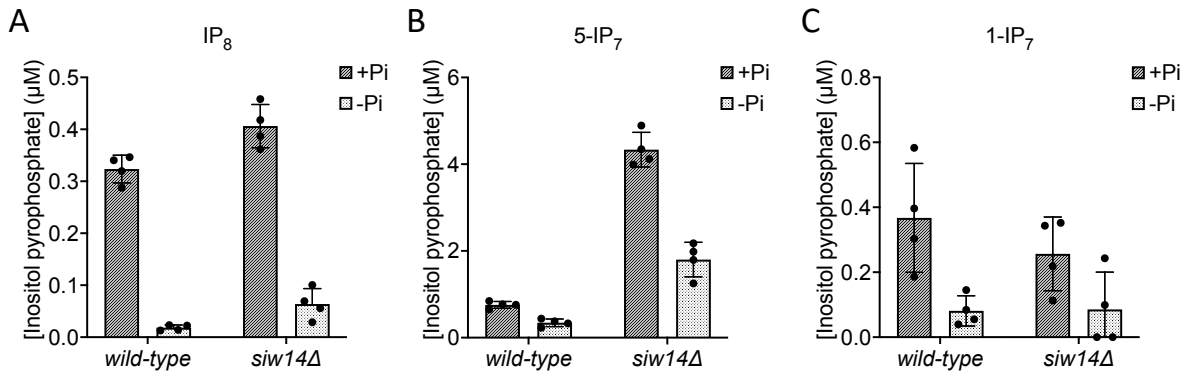


Fig. S4: Loss of PP-IPs from *siw14Δ* cells upon P_i starvation.

The indicated strains were grown logarithmically in SC medium containing 7.5 mM of P_i (30°C, 150 rpm, overnight). The cells were spun down, washed twice with P_i starvation medium and further incubated in P_i starvation medium or in SC with Pi for 2h. The inoculum for the samples for this final 2h incubation was adjusted such that all samples had an OD_{600nm} of 1 (10^7 cells/ml) at the time of harvesting. After the two hours incubation, 1 ml of culture was extracted with perchloric acid and analyzed for PP-IPs by CE-ESI-MS. The data was normalized by the number of cells harvested before calculating cytosolic concentrations. The y-axis provides the estimated cytosolic concentrations based on an average cell volume of 42 fL. Means (n=4) and standard deviations are indicated.

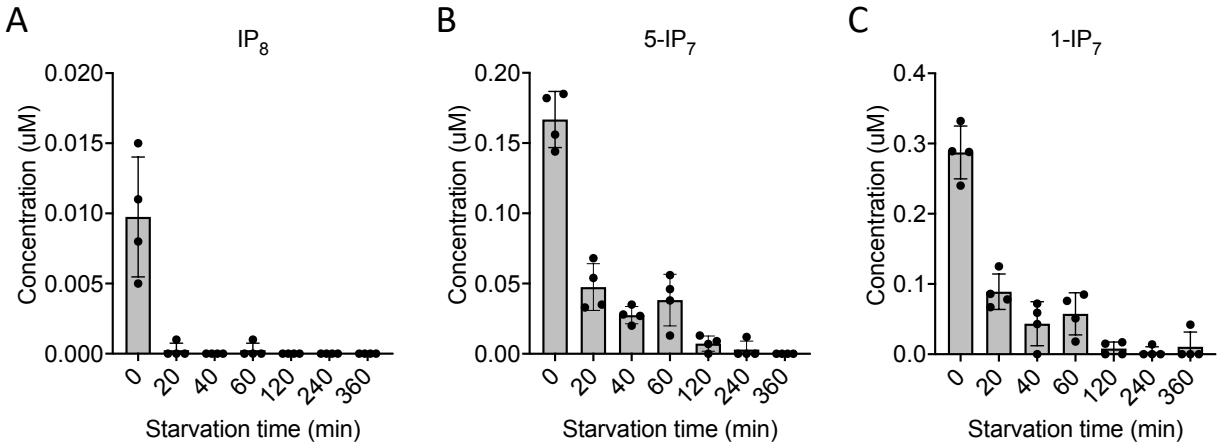


Fig. S5: Inositol pyrophosphate analysis in C. neoformans

Cells were logarithmically grown in synthetic complete (SC) medium for 17h up to an OD_{600nm} of 1. Cells were sedimented by centrifugation, resuspended in SC without P_i, and incubated further. At the indicated times, aliquots were extracted with perchloric acid. PP-IPs were enriched on TiO_2 beads and analyzed by CE-MS. Concentrations of 1,5-IP_8, 5-IP_7 and 1-IP_7 in the extracts were determined by comparison with added synthetic ^{13}C-labeled PP-IP standards. The graphs provide the concentrations in the extracts. n=4; means and standard deviations are indicated. The IPP analysis data were normalized to the OD_{600} of the culture at the given time point.