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The SPX domain of the yeast low-affinity phosphate transporter Pho90 regulates transport activity

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Yeast has two phosphate-uptake systems that complement each other: the high-affinity transporters (Pho84 and Pho89) are active under phosphate starvation, whereas Pho87 and Pho90 are low-affinity transporters that function when phosphate is abundant. Here, we report new regulatory functions of the amino-terminal SPX domain of Pho87 and Pho90. By studying truncated versions of Pho87 and Pho90, we show that the SPX domain limits the phosphate-uptake velocity, suppresses phosphate efflux and affects the regulation of the phosphate signal transduction pathway. Furthermore, split-ubiquitin assays and co-immunoprecipitation suggest that the SPX domain of both Pho90 and Pho87 interacts physically with the regulatory protein Spl2. This work suggests that the SPX domain inhibits low-affinity phosphate transport through a physical interaction with Spl2.

Keywords: metabolism; phosphate; SPX domain; transporter; yeast

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INTRODUCTION

Transmembrane transporters are required for the uptake and accumulation of nutrients from dilute environments, and generate the concentration gradients of ions and metabolites that are essential for life itself (Saier, 2000). Therefore, transport activity must be adjusted to changing intracellular and extracellular conditions. In *Saccharomyces cerevisiae,* phosphate homeostasis is regulated by the phosphate signal transduction (PHO) pathway: under phosphate limitation, transcription of the high-affinity phosphate transporter genes *PHO84* and *PHO89* (as well as many other genes) is upregulated, which leads to increased phosphate uptake and consecutive downregulation of the PHO pathway (Lenburg & O'Shea, 1996; Ogawa *et al*, 2000; Wykoff & O'Shea, 2001). The PHO pathway also upregulates the transcription of *SPL2*, which inhibits the low-affinity phosphate-uptake system and thus causes positive feedback on the PHO pathway. This regulatory mechanism results in cells that exclusively use either the high-affinity or the low-affinity phosphate-transport system depending on the phosphate supply (Wykoff *et al*, 2007).

The low-affinity transporters Pho87 and Pho90 are >60% identical (based on the amino-acid sequence) and have been implicated in regulatory functions that go beyond the uptake of phosphate from the environment (Auesukaree *et al*, 2003; Giots *et al*, 2003; Pinson *et al*, 2004; Hürlimann *et al*, 2007). A feature of Pho87 and Pho90 that could mediate such functions is the amino-terminal SPX domain (named after yeast Syg1 and Pho81 and human XPR1, PF03105, http://pfam.sanger.ac.uk/). SPX domains are present at the N-termini of various proteins in eukaroytes and are thought to exert regulatory functions through G proteins (Spain *et al*, 1995; Barabote *et al*, 2006). Apart from Pho87 and Pho90, many other fungal and plant proteins involved in phosphate metabolism, as well as human transporters that act as xenotropic and polytropic murine leukaemia virus receptors, feature such an N-terminal domain (Battini *et al*, 1999; Tailor *et al*, 1999; Yang *et al*, 1999; Tian *et al*, 2007; Wang *et al*, 2008). However, none of these SPX domains has been characterized biochemically, and their biological functions remain unknown. This study aimed to identify the functions of the SPX domain in the low-affinity phosphate transporters Pho87 and Pho90 in yeast.

RESULTS AND DISCUSSION

To identify the functions of the SPX domain of Pho87 and Pho90, we created N-terminal truncations directly in the genome of *S. cerevisiae* (Fig 1A). The 375 amino acids that were thus removed in the N-terminal truncations comprise the entire SPX domain. All studies presented here were carried out on Pho90, but most conclusions were strengthened by showing similar results in Pho87 (supplementary information online).

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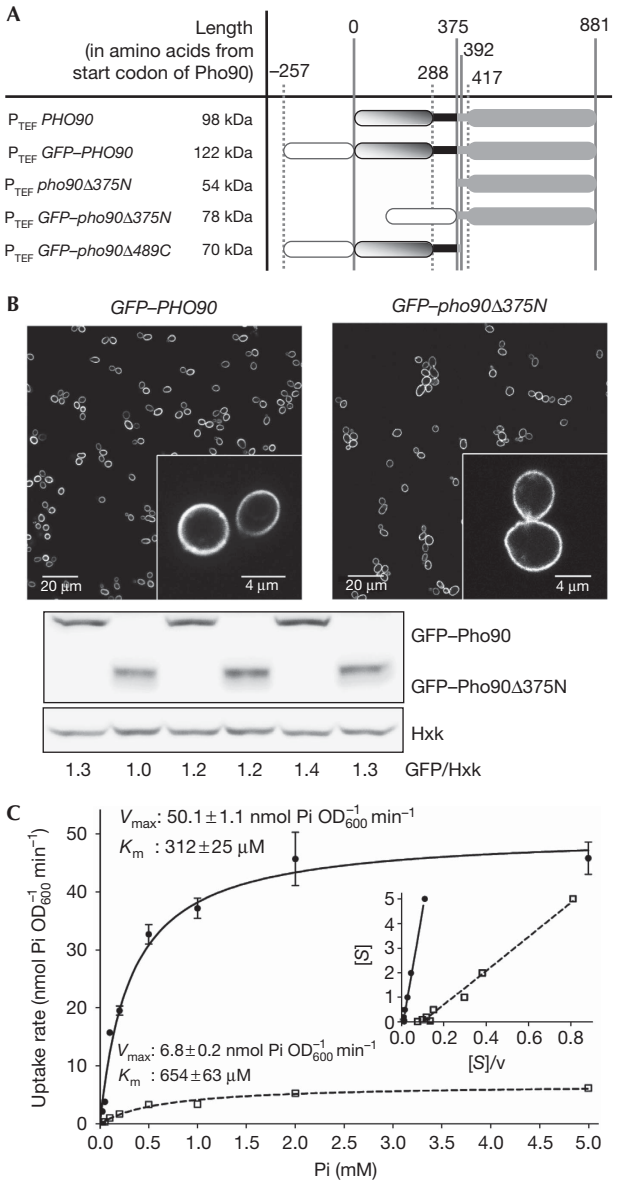

Fig 1 | The SPX domain of Pho90 inhibits phosphate uptake. (**A**) To identify the functions of the SPX domain of Pho87 and Pho90, we created amino-terminal truncations directly in the genome of *Saccharomyces cerevisiae*. (**B**) N-terminally GFP-tagged Pho90 (FFSc519) and Pho90Δ375N (FFSc567) are both present at similar levels and localize to the plasma membrane. (**C**) Phosphate uptake in strains lacking all phosphate transporter genes except full-length *PHO90* (open squares, FFSc926) or *pho90Δ375N* (filled circles, FFSc931), which are both overexpressed using the TEF promoter, in the EY920 background. Each point represents the average and s.d. of at least three uptake experiments. Inset: Hanes–Woolf plot ([*S*]: mMPi; v; nmol Pi OD_{600}^{-1} min^{-1}) GFP, green fluorescent protein; Hxk, hexokinase; Pho, phosphate signal transduction; TEF, transcriptional enhancer factor.

The SPX domain of Pho90 regulates phosphate uptake

Protein levels and plasma membrane localization of Pho90 were not affected by the removal of the SPX domain (375-amino-acids N-terminal truncation), thus suggesting that Pho87 and Pho90 levels and localization were both independent of an N-terminal signal sequence (Fig 1B and supplementary Fig S1 online). However, the strain expressing the truncated transporter (*pho90Δ375N*) and lacking all other phosphate transporters showed a higher phosphate-uptake rate (50.1 nmol Pi OD_{600}^{-1} min^{-1}) as compared with the control strain (6.8 nmol Pi OD_{600}^{-1} min^{-1}), although the K_m (312 and 654 μM, respectively) remained in the same range as published earlier (Fig 1C; 205 μM, Wykoff & O'Shea, 2001). It was thus concluded that the truncated phosphate transporters have increased catalytic activity.

The *pho90Δ375N* strain also showed higher total phosphate (P_{tot}) and polyphosphate (poly P) content as compared with the strain overexpressing the full-length protein (Table 1, supplementary Fig S2 online). This finding suggests a broader effect of the SPX domain on phosphate metabolism because P_{tot} and poly P levels are cumulative measures that result from the integration of uptake, allocation, storage and efflux of phosphate over time. Surprisingly, strains containing the Pho87- or Pho90-N-terminal truncation, but not the control strains, arrested growth on a medium containing 50 mM phosphate (Fig 2A and supplementary Fig S3 online; standard synthetic defined (SD) medium contains 7.4 mM phosphate). This growth arrest was observed with phosphate concentrations starting from 10 mM and was neither pH-dependent (data not shown) nor potassium-dependent (50 mM KCl; supplementary Fig S3 online). The higher the phosphate availability, the more it was accumulated by the *pho90Δ375N* strain, whereas the control strains did not increase internal phosphate content at external phosphate concentrations greater than 2 mM (Fig 2B). The SPX domain of Pho90 could thus be considered as an auto-inhibitory domain that is required to regulate and restrict phosphate accumulation.

Spl2 interacts directly with Pho90

The study by Wykoff *et al* (2007) shows that Spl2 inhibits the low-affinity phosphate-uptake system by an unknown mechanism. We wanted to test whether this regulation was mediated through the N-terminal SPX domain. Overexpression of *SPL2* reduced phosphate uptake in strains overexpressing full-length *PHO90* (initial velocities decreased from 5.8 ± 0.3 to 1.6 ± 0.2 nmol Pi min^{-1} OD_{600}^{-1}), but had no effect on the uptake in strains lacking the Pho90 SPX domain (25 ± 5 and 26 ± 4 nmol Pi min^{-1} OD_{600}^{-1} without and with P_{TEF} *SPL2*, respectively; supplementary Fig S4 online). This suggests that the Pho90 SPX domain is required for the Spl2-dependent inhibition of low-affinity phosphate uptake. Although P_{tot} content and poly P levels are the consequences not only of phosphate uptake, but also of phosphate utilization, storage and efflux, these measures showed similar behaviour. The overexpression or deletion of *SPL2* (in the P_{TEF} *PHO90* strain) correlated with reduced and increased P_{tot} and poly P contents, respectively (Table 1, supplementary Fig S5 online). This correlation between the presence and absence of Spl2 with the P_{tot} and poly P levels was much clearer in the strain background containing only Pho90 as phosphate transporters (supplementary Fig S5 online). Interestingly, overexpression of *SPL2* in the *pho90Δ375N* strain caused a slight increase in P_{tot} and poly P levels and a reduction in cytosolic Pi concentrations (Table 1). Thus, Spl2 must have additional effects on phosphate allocation and metabolism that do not depend on the Pho90 SPX domain, possibly through the interaction with other SPX domains.

Table 1 | Removal of the Pho90 SPX domain leads to unrestricted phosphate accumulation independent of the presence of Spl2 but has no effect on cytosolic Pi levels

Strain	Pi cyto	Poly P	P_{tot}	Poly P
	(nmol Pi per mg dry weight)		(μg Pi OD_{600}^{-1})	(μg poly P OD_{600}^{-1})
WT (BY4741)	72 ± 2	199 ± 11	20 ± 2	1.7 ± 0.2
PHO90 (FFSc701)	60 ± 3	352 ± 24	26 ± 5	2.8 ± 0.5
pho90Δ375N (FFSc574)	63 ± 10	608 ± 44	54 ± 9	10.5 ± 2.6
SPL2 (FFSc754)	64 ± 3	123 ± 15	20 ± 3	1.1 ± 0.1
PHO90 SPL2 (FFSc762)	63 ± 1	134 ± 11	17 ± 2	1.2 ± 0.2
pho90Δ375N SPL2 (FFSc764)	47 ± 5	528 ± 3	59 ± 10	14.1 ± 2.8
spl2Δ	60 ± 2	203 ± 9	20 ± 1	1.2 ± 0.3
PHO90 spl2Δ (FFSc873)	61 ± 2	367 ± 20	27 ± 2	2.8 ± 0.4
pho90Δ375N spl2Δ (FFSc877)	75 ± 1	525 ± 11	50 ± 4	7.1 ± 0.7

Cytosolic (Pi cyto) and poly P content were determined by ^{31}P nuclear magnetic resonance spectroscopy (indicated in nmol Pi per mg dry weight). Poly P content was also determined by extraction and specific digestion (see Methods) and P_{tot} was quantified after acid hydrolysis (both given in μg *X* OD_{600}^{-1}). All values represent the average and s.d. of three measurements. Pho, phosphate signal transduction; WT, wild type.

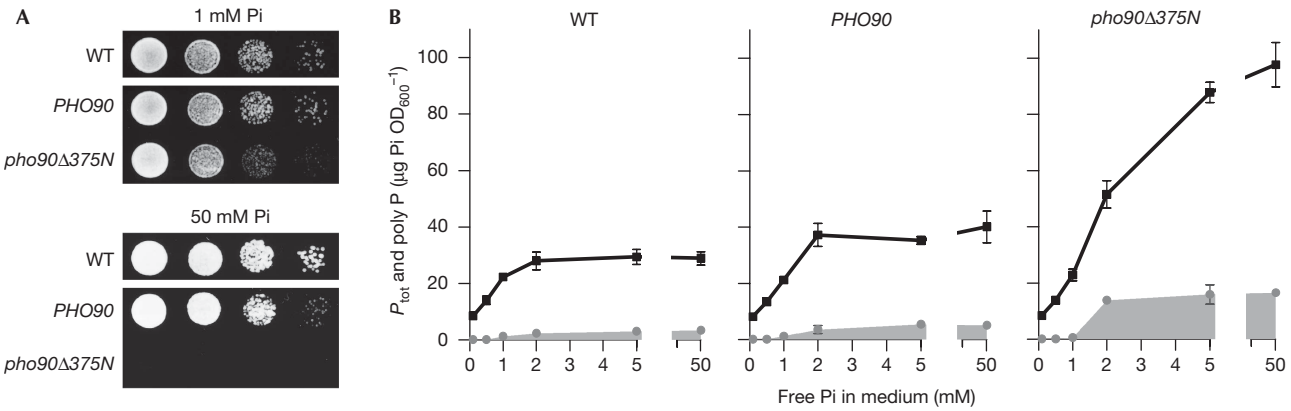

Fig 2 | The Pho90 SPX domain is essential for normal growth on high-phosphate medium and limits phosphate accumulation. All strains (WT (BY4741), P_{TEF} *PHO90* (FFSc701) and P_{TEF} *pho90Δ375N* (FFSc574)) were precultured in YPD medium. (**A**) After dilution to an OD_{600} of 0.3, 10 × dilution series were spotted on SD medium containing 1 or 50 mM phosphate. (**B**) Yeast cells were transferred to YPD medium, which was adjusted to the phosphate concentrations indicated on the abscissa and P_{tot} (black line) and poly P (grey area) were measured. Each data point represents the average and s.d. of four replicates. GFP, green fluorescent protein; Pho, phosphate signal transduction; SD, synthetic defined; TEF, transcriptional enhancer factor; WT, wild type; YPD, yeast extract-peptone-dextrose.

On the basis of these results for phosphate uptake, P_{tot} and poly P levels, we hypothesized that Spl2 is bound directly by the SPX domain of Pho90. The physical interaction of Spl2 with Pho90 was confirmed independently by using the split-ubiquitin assay (Fig 3A and supplementary Fig S6 online) and by using immunoprecipitation of Spl2 with a soluble SPX domain fused to green fluorescent protein (GFP; supplementary Fig S7 online). We thus suggest that the SPX domain of Pho90 is required for the Spl2-dependent inhibition of phosphate uptake and hypothesize that this regulation arises from the physical interaction between the Pho90 SPX domain and Spl2.

The SPX domain prevents phosphate efflux

Earlier results (Wykoff *et al*, 2007) show that *S. cerevisiae* evolved a complex mechanism for the inhibition of the low-affinity phosphate-uptake system under phosphate limitation, which seems counterintuitive. We therefore tested whether yeast cells lose phosphate in a Pho90-, SPX- and Spl2-dependent manner.

Under the conditions tested, all strains leaked phosphate to the medium (Fig 4 and supplementary Fig S8 online). The amount of phosphate released from phosphate-laden yeast cells was highest in the cells lacking the Pho90 SPX domain and the strain overexpressing *PHO90*, and did not correlate with the poly P stores (*pho85Δpho91Δ*: poly P high, efflux low; *pho90Δ375N*: poly P high, efflux high; Fig 4 and supplementary Fig S8 online). Overproduction of Spl2 reduced the phosphate release of the *PHO90* strain by a factor of 6, whereas it had no effect in the strain lacking the SPX domain of Pho90 (Fig 4). These data suggest that Pho90 can mediate phosphate efflux if not regulated

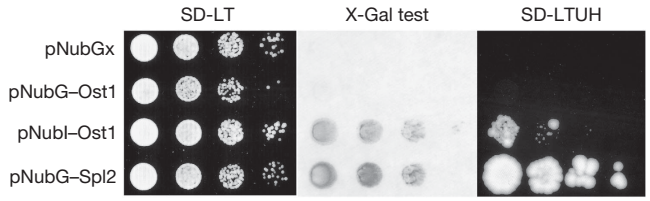

Fig 3 | The split-ubiquitin assay suggests a physical interaction between Spl2 and Pho90. The strains (L40ccua background) were spotted (in 10× dilutions) on SD-Leu–Trp (as a control and for the X-*gal* reporter assay) and on SD-His–Leu–Trp–Ura medium (to assess the interaction). Both reporter assays suggested an interaction of the bait Pho90 (pNCW–Pho90) with the positive control (pNubI–Ost1) and with Spl2 (pNubG–Spl2), but not with the empty prey vector (pNubGx) or the negative control (pNubG–Ost1). Pho, phosphate signal transduction; SD-LT, synthetic defined-Leu–Trp; SD-LTUH, synthetic defined-His–Leu–Trp–Ura.

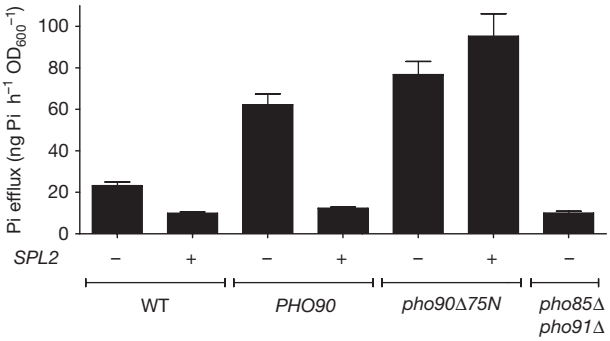

Fig 4 | The SPX domain of Pho90 and Spl2 regulate phosphate efflux. Different strains (BY4741 background) expressing either full-length or truncated *PHO90* using the TEF promoter were precultured in YPD medium for 4 h (high intracellular phosphate content) and transferred to phosphate-free medium. Overexpression (from the TEF promoter) or wild-type regulation of *SPL2* are indicated by '+' and '−' signs, respectively. The phosphate content in the supernatant medium was monitored after 2 h. As a control, we included the *pho85Δpho91Δ* strain, which contains high poly P levels and shows little phosphate efflux. The bars represent the average and s.d. of four measurements. Pho, phosphate signal transduction; TEF, transcriptional enhancer factor; WT, wild type; YPD, yeast extract-peptone-dextrose.

by Spl2 and the SPX domain. It can therefore be hypothesized that the regulation of *SPL2* by the PHO pathway evolved to minimize phosphate efflux through low-affinity transporters under phosphate starvation.

The Pho90 SPX domain fine-tunes the PHO pathway

Finally, we studied the consequences of Pho90 SPX-domain removal on the transcriptional regulation of the PHO pathway. As expected from the increased phosphate uptake (Fig 1C), overexpression of either full-length or N-terminally truncated *PHO90* led to a marked repression of PHO-pathway transcription (Fig 5). This transcriptional downregulation of the PHO pathway correlated with the increase in P_{tot} and poly P contents, but was not related to cytosolic Pi levels (Table 1). Consequently, neither poly P nor cytosolic Pi levels can be signals for PHO-pathway-dependent gene regulation, and another organic phosphate, such as inositol pyrophosphate, must be involved (Lee *et al*, 2007).

In addition, simultaneous overexpression of full-length *PHO90* and *SPL2* abolished the transcriptional repression of the PHO pathway and reverted the P_{tot} and poly P levels to those of the P_{TEF} *SPL2* strain (Fig 5 and Table 1). By contrast, overexpression of both *pho90Δ375N* and *SPL2* neither restored normal PHO transcription nor wild-type P_{tot} and poly P contents (Fig 5 and Table 1). Altogether, these data suggest that the interaction between the Pho90 SPX domain and Spl2 mediates fine-tuning between phosphate uptake, phosphate storage and phosphate utilization, which is ensured by the PHO pathway and the two phosphate-uptake systems.

Conclusion

This study documents a new *cis*-regulatory function of the Pho87 and Pho90 SPX domain in yeast. The SPX domain regulates phosphate-uptake activity and prevents phosphate efflux through Pho87 and Pho90. We further show that the PHO-regulated protein Spl2 interacts with the SPX domain of Pho87 and Pho90 and thereby contributes to regulatory functions. Under low phosphate availability, the PHO pathway is active, the *SPL2* gene is upregulated, Pho87 and Pho90 are inhibited, and phosphate efflux is prevented. In stationary phase, this inability to prevent phosphate efflux (in the strain lacking the Pho90 SPX domain) also caused a marked decrease in the cellular phosphate content to levels even below those of the wild type (data not shown).

Under normal or high phosphate availability, the PHO pathway is inactive and transcription of *SPL2* is repressed, but the Pho87 and Pho90 SPX domains still regulate phosphate transport. In normal yeast extract-peptone-dextrose (YPD) medium, strains lacking the SPX domain of either Pho87 or Pho90 showed strongly increased P_{tot} and poly P content. At phosphate levels above 10 mM, deletion of the Pho87 or Pho90 SPX domain caused unrestricted phosphate uptake and increased phosphate accumulation until cells were no longer able to grow. The Pho87 and Pho90 SPX domain must therefore regulate phosphate transport and efflux independent of the PHO pathway and of Spl2.

On a much broader scale, similar *cis*-regulatory mechanisms as the ones described here might also exist for the SPX homologues in other yeast proteins, plants and mammalian retrovirus receptors. Interestingly, most retroviral receptors are transmembrane transporters of molecules such as amino acids, inorganic phosphate, myo-inositol or thiamine (Hein *et al*, 2003; Tailor *et al*, 2003; Collins *et al*, 2004; Mendoza *et al*, 2006). The SPX domain of mammalian retrovirus receptors could therefore function as a regulatory domain as it does in the yeast phosphate transporters.

METHODS

Yeast strains and cultivation. Yeast strains are listed in supplementary Table S1 online. Strains were routinely grown at 30 °C in either SD medium (6.8 g l^{-1} yeast nitrogen base, complete supplement or drop-out mix (from Qbiogene, Solon, OH, USA or Formedium, Hunstanton, UK), 20 g l^{-1} glucose) or YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} glucose), with

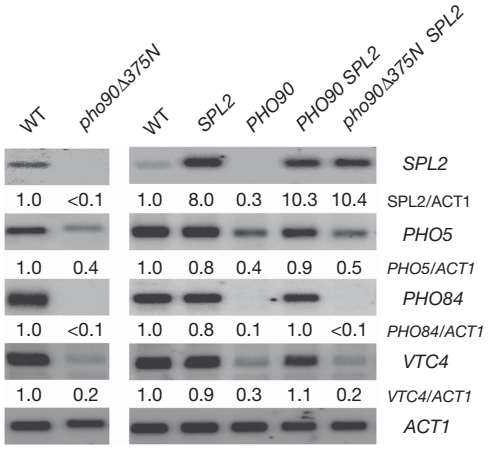

Fig 5 | Overexpression of *SPL2* abolishes the downregulation of the PHO pathway in the P_{TEF} *PHO90* strain only if the Pho90 SPX domain is present. All strains (WT (BY4741), P_{TEF} *PHO90* (FFSc701), P_{TEF} *pho90Δ375N* (FFSc574), P_{TEF} *SPL2* (FFSc754), P_{TEF} *PHO90* P_{TEF} *SPL2* (FFSc762) and P_{TEF} *pho90Δ375N* P_{TEF} *SPL2* (FFSc764)) were cultured in YPD medium and harvested after 4 h (late exponential phase). Transcript levels of the PHO marker genes *SPL2*, *PHO5*, *PHO84* and *VTC4* (as compared with the actin control) were quantified with a phosphorimager from two or three independent northern blots for which mean variations were below 10% of the ratio values. One representative northern blot is shown. PHO, phosphate signal transduction; TEF, transcriptional enhancer factor; WT, wild type.

the optional addition of 200 mg l^{-1} G418 (PAA Laboratories GmbH, Pasching, Austria) or 100 mg l^{-1} clonNat (Werner BioAgents, Jena, Germany). The strains EY57 and EY920 were maintained on YPGal medium (YPD medium containing 20 g l^{-1} galactose as a carbon source; Wykoff & O'Shea, 2001). Low-phosphate YPD medium was prepared as described previously (Kaneko *et al*, 1982; Werner *et al*, 2005).

Manipulation of yeast strains. Yeast genomic DNA was isolated by using the 'smash-and-grab' protocol (Rose *et al*, 1990). Plasmids were transformed by the method of Gietz *et al* (1992). Selection markers were exchanged and additional genes were deleted, truncated or tagged as described by Janke *et al* (2004). All strains were verified by PCR. Pho90- and Pho87-N-terminal truncations were obtained by homologous recombination directly in the genome of *S. cerevisiae* (without the addition of new coding sequence except for new start or stop codons, Fig 1A). At the same time, we exchanged the native promoter with the constitutive transcriptional enhancer factor (TEF) promoter (from the *TEF1* gene).

Quantification of poly P and P_{tot} contents. P_{tot} and poly P were determined and quantified as described previously (Werner *et al*, 2005; Freimoser *et al*, 2006). With the help of a phosphate standard, P_{tot} and poly P levels were calculated by using 97 g mol^{-1} (H_2PO_4) and 80 g mol^{-1} (molecular weight of the HPO_3 residues in poly P), respectively.

Phosphate-uptake measurements. Yeast cells were grown overnight in YPD ($OD_{600} \approx 4$–8), inoculated in fresh medium ($OD_{600} \approx 0.25$), harvested ($OD_{600} \approx 0.6$–1) and washed twice (phosphate-free SD medium, 4% glucose with centrifugation at 1,500 *g*, 4 °C, 5 min). Cells were resuspended in the phosphate-free medium ($OD_{600} \approx 20$) and kept at 4 °C. Before the uptake, experimental cells were shaken at 24 °C for 5 min. A 10× phosphate solution (250 μM–10 mM with 3.8–4.5 × 10^6 counts per minute (CPM), or 20 and 50 mM with 8 × 10^6 CPM) was added to 0.9 volumes of the cell suspension; uptake was stopped by filtering and washing with 5 ml of a 500 mM phosphate buffer. Radioactivity was quantified by scintillation counting, the amount of phosphate that was taken up was calculated, and V_{max} and K_m were determined by non-linear curve fitting (Prism 5, GraphPad Software, La Jolla, CA, USA).

Phosphate efflux assay. Cells were precultured overnight in YPD, transferred to fresh medium ($OD_{600} \approx 1$) and harvested after 4 h. After two washing steps (phosphate-free SD medium, 2% glucose), cells were resuspended in the same medium ($OD_{600} \approx 20$). The phosphate released into the medium was quantified after 2 h by the malachite green assay as described previously (Werner *et al*, 2005).

^{31}P nuclear magnetic resonance spectroscopy. Yeast strains were grown overnight in 100 ml YPD medium and were harvested at $OD_{600} \approx 3$–4.5. ^{31}P nuclear magnetic resonance spectroscopy was carried out as described previously (Pinson *et al*, 2004), and the measurements for cytosolic orthophosphate and poly P are given as millimolar of orthophosphate residues.

Split-ubiquitin assay. The bait and prey constructs were generated with the vectors pNCW and pNubGx and used according to Iyer *et al* (2005). We used the *lacZ* reporter and the two auxotrophic markers *HIS3* and *URA3* to assess the interaction.

Northern blot analysis. Transcript levels of *SPL2*, *PHO5*, *PHO84*, *VTC4* and *ACT1* were determined by northern blot analysis as described previously (Pinson *et al*, 2004).

Western blot analysis. Proteins from cells expressing *GFP–PHO90* or *GFP–pho90Δ375N* were extracted, separated by SDS–PAGE gel electrophoresis and blotted as described previously (Horak & Wolf, 2001). The blots were hybridized with GFP (1:4,000, Living colors A.v. Monoclonal Antibody (JL-8), Clontech, Mountain View, CA, USA) and Hxk antibodies (Rockland Immunochemicals Inc., Gilbertsville, PA, USA), scanned (FluorChem SP, Alpha Innotech Corp., San Leandro, CA, USA), and protein levels were quantified by peak area integration of three independent blots.

Confocal microscopy. Localization of GFP-tagged Pho90 and Pho90Δ375N was carried out as described previously by Hürlimann *et al* (2007).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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