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## Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in *Saccharomyces cerevisiae*

Ruben Ghillebert, Erwin Swinnen, Pepijn de Snijder, Bart Smets, Joris Winderickx

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1 **Differential roles for the low-affinity phosphate transporters Pho87 and**  
2 **Pho90 in *Saccharomyces cerevisiae***

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7 **Ruben Ghillebert<sup>1</sup>, Erwin Swinnen<sup>1</sup>, Pepijn De Snijder, Bart Smets and Joris**  
8 **Winderickx\***

9  
10  
11  
12  
13 **Functional Biology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 31, Box**  
14 **2433, B-3001 Heverlee, Belgium**

15  
16  
17  
18  
19 **<sup>1</sup> these authors contributed equally and should be considered co-first authors**

20  
21  
22  
23  
24 **\* for correspondence: [joris.winderickx@bio.kuleuven.be](mailto:joris.winderickx@bio.kuleuven.be); Tel. :+32-16-321516; Fax +32-**  
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35 **targeting, non-redundancy**

37 **Summary**

38

39 **When starved for phosphate (Pi), yeast cells activate the PHO-signaling pathway,**  
40 **wherein the Pho4 transcription factor mediates expression of genes involved in Pi-**  
41 **acquisition, such as *PHO84*, encoding the high-affinity H<sup>+</sup>/Pi-symporter. In contrast,**  
42 **transcription of *PHO87* and *PHO90*, encoding the low-affinity H<sup>+</sup>/Pi-transport system, is**  
43 **independent of phosphate status. Here, we reveal that upon Pi-starvation, these low-**  
44 **affinity phosphate transporters are endocytosed and targeted to the vacuole. For Pho87,**  
45 **this process strictly depends on *SPL2*, another Pho4-dependent gene that encodes a**  
46 **protein known to interact with the N-terminal SPX-domain of the transporter. In**  
47 **contrast, the vacuolar targeting of Pho90 upon Pi-starvation is independent of both**  
48 **Pho4 and Spl2, though it still requires its SPX-domain. Furthermore, both Pho87 and**  
49 **Pho90 are also targeted to the vacuole upon glucose starvation or upon treatment with**  
50 **rapamycin, which mimics nitrogen-starvation, but, while these responses are**  
51 **independent of PHO-pathway signaling, they again require the N-terminal SPX-domain**  
52 **of the transporters. These observations suggest that other SPX-interacting proteins must**  
53 **be involved. In addition, we show that Pho90 is the most important Pi-transporter under**  
54 **high Pi conditions in the absence of a high-affinity Pi-transport system. Taken together,**  
55 **our results illustrate that Pho87 and Pho90 represent non-redundant Pi-transporters,**  
56 **which are tuned by the integration of multiple nutrient signaling mechanisms in order to**  
57 **adjust Pi-transport capacity to the general nutritional status of the environment.**

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## 59 Introduction

60 As inorganic phosphate (Pi) is an essential nutrient for all organisms, maintaining proper Pi  
61 homeostasis is critical to ensure survival of cells and organisms. A crucial step in controlling  
62 Pi homeostasis encompasses the transport of Pi across the plasma membrane into the cell.  
63 Since cellular membranes are impermeable to Pi, specialised integral membrane proteins are  
64 required to transport this essential compound. In *Saccharomyces cerevisiae*, two Pi-symport  
65 systems exist [1-3]. The low-affinity transport system has a  $K_m$  for external Pi of  
66 approximately 1 mM and satisfies the cellular need for Pi at normal or high Pi concentrations.  
67 This low-affinity Pi-transport system is believed to be composed of the partially redundant  
68 Pho87 and Pho90 H<sup>+</sup>/Pi-symporters [4]. A third gene, *PHO91*, was initially considered to be a  
69 third component of the low-affinity plasma membrane phosphate transport system [5], but  
70 later studies localized the Pho91 protein at the vacuolar membrane, indicating that this protein  
71 is not directly involved in the acquisition of phosphate from the external medium [6]. The  
72 high-affinity Pi-transport system has a  $K_m$  of about 10  $\mu$ M and is sustained by the Pho84 and  
73 Pho89 high-affinity H<sup>+</sup>/Pi- and Na<sup>+</sup>/Pi-symporters [7, 8]. While expression of the *PHO87*,  
74 *PHO90* and *PHO91* genes was shown to be independent of Pi concentrations, transcription of  
75 *PHO84* and *PHO89* is induced when Pi becomes limiting, a process controlled by the PHO-  
76 pathway [9]. Signal flow through this PHO-pathway is regulated by the cyclin-dependent  
77 kinase inhibitor (CKI) Pho81. Under Pi-starving conditions, activation of this CKI leads to  
78 inhibition of the Pho85-Pho80 cyclin-dependent kinase complex and nuclear translocation of  
79 the dephosphorylated transcription factor Pho4. Nuclear Pho4 then induces a set of genes  
80 known as the PHO-genes [10], which includes *PHO84* and *PHO89*. When sufficient amounts  
81 of extracellular Pi are available, Pho81 is inactivated and this results in an active Pho85-  
82 Pho80 kinase complex that is able to phosphorylate and trigger the nuclear exclusion of Pho4,  
83 with as consequence the repression of the PHO-genes [11].

84  
85 An additional role for the Pho85-Pho80 complex is to regulate the localization and hence the  
86 activation of the Rim15 protein kinase in response to fluctuating Pi levels [11]. Rim15 is  
87 essential for proper entry into stationary phase as it controls the activity of the transcription  
88 factors Msn2,4 and Gis1, which are required for expression of the so-called Stress-  
89 Responsive-Element (STRE) genes and the Post-Diauxic-Shift (PDS) genes, respectively [12-  
90 14]. Rim15 acts as a nutritional integrator in yeast, since it is regulated by at least four  
91 nutrient-regulated kinases: PKA inhibits Rim15 activity [15], while TORC1 and Sch9  
92 promote cytoplasmic retention [16, 17] and the Pho85-Pho80 complex promotes its nuclear  
93 exclusion [11, 18]. Under Pi-starving conditions, inhibition of the Pho85-Pho80 kinase by  
94 Pho81 results in an active, nuclear Rim15 and proper entry into stationary phase. In contrast,  
95 under high Pi conditions, phosphorylation of Rim15 by the Pho85-Pho80 kinase induces its  
96 nuclear exclusion thereby leading to its inactivation. In line with this, we already showed that  
97 the Pho85-Pho80 complex mediates Pi-dependent activation of typical PKA-targets like  
98 activation of trehalase, mobilisation of trehalose and repression of stress-responsive genes and  
99 that the regulation of many of these properties occurs via a Pho85-Pho80-dependent inhibition  
100 of Rim15 activity [19].

101  
102 The ability of inorganic Pi to act as a nutrient signal affecting PKA targets is closely  
103 correlated to its uptake into the cell, as inactivation of Pi-transport by destruction of the  
104 proton gradient across the plasma membrane with the protonophore 2,4-dinitrophenol  
105 completely prevented Pi-induced trehalase activation [20]. Although the predominant Pi-  
106 transport activity present in Pi-starved cells is represented by the Pho84 transporter [7, 21],  
107 which is additionally exemplified by the inability of a *pho84 $\Delta$*  strain to mediate rapid Pi-  
108 signaling [22, 23], we previously observed clear activation of trehalase after addition of Pi to

109 cells deleted for *PHO4* or *PHO81*, in which no *PHO84* transcription can be detected [19].  
110 Additionally, both Pho84 and Pho87 have been shown to act as specific Pi sensors for rapid  
111 Pi-signaling [20], but the genetic and physiological relevance of Pi-signaling through Pho87  
112 was never investigated. Our data reveals that in the absence of Pho4, the low affinity Pi-  
113 transporter Pho87 becomes essential for Pi-signaling. We show that in Pi-starved cells, the  
114 low-affinity Pi-transporters Pho87 and Pho90 are normally subjected to vacuolar targeting,  
115 but in cells devoid of Pho4 activity, this process is specifically hampered for Pho87 due to the  
116 lack of expression of *SPL2*, which was previously identified as a negative regulator of low-  
117 affinity Pi-transport and was shown to interact with the N-terminal SPX-domain of both  
118 Pho87 and Pho90 [4, 24]. However, we now demonstrate that, in contrast to Pho87, the  
119 vacuolar targeting of the Pho90 upon Pi-starvation is independent of both Pho4 activity and  
120 Spl2. Furthermore, when cells are starved for other essential nutrients or are treated with  
121 rapamycin, the vacuolar targeting applies to both low-affinity Pi-transporters and also this  
122 process is independent of Pho4 and Spl2. Nonetheless, the vacuolar targeting of both Pho87  
123 and Pho90 under these conditions still depends on their N-terminal SPX-domain. This  
124 indicates that besides Spl2, other, yet unidentified, proteins must interact with this domain in  
125 order to fine-tune the presence of the low-affinity Pi-transporters at the plasma membrane in  
126 response to a multitude of nutrient signals.  
127

## 128 **Experimental procedures**

### 129 *Strains*

130 *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Deletions were made  
131 using polymerase chain reaction-based disruption cassettes as described previously [25].  
132 Yeast was transformed with the Gietz method [26]. Sporulation was performed by spotting  
133 diploid cells on plates containing 1% K-acetate, 0.1% KHCO<sub>3</sub>, pH 6.0 for 5 to 6 days at 24°C.

134 Tetrads were dissected and separated in a raster using a micromanipulator (Singer  
135 Instruments). The P<sub>TEF</sub>-EGFP-*PHO87*, P<sub>TEF</sub>-EGFP-*PHO90*, P<sub>TEF</sub>-EGFP-*PHO87Δ375N* and  
136 P<sub>TEF</sub>-EGFP-*PHO90Δ375N* were kindly provided by Hurlimann *et al.* [4, 6].

137

### 138 *Culture and starvation conditions*

139 Yeast cells were grown in synthetic complete minimal medium containing 0,5% w/v  
140 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1,9g/l yeast nitrogen base w/o amino acids, supplemented with all amino acids  
141 and 2% w/v glucose (ScD). For monitoring growth on ScD agar with fluctuating extracellular  
142 Pi levels, a preculture on ScD was inoculated in fresh ScD medium at an initial OD<sub>600</sub> of 0.1  
143 and tenfold dilutions were spotted on phosphate starvation medium (5.7 g/l of yeast nitrogen  
144 base with ammonium sulphate without phosphate) with 2% glucose, required amino  
145 acid/nucleotide supplements and with known Pi concentration. Pictures were taken after 2-3  
146 days. For growth experiments with arsenate, cells were grown in standard rich medium with  
147 2% w/v glucose (YPD) and spotted on YPD or on YPD with an additional 10mM Pi and with  
148 or without 5mM AsO<sub>4</sub>. For phosphate signaling and consumption experiments, yeast cells  
149 were grown into mid-exponential phase (OD<sub>600</sub> = 1.5-2) on ScD medium, harvested, washed  
150 two times and transferred to phosphate starvation medium with 4% glucose and required  
151 amino acid/nucleotide supplements. Cells were starved for phosphate for 3 days, during which  
152 starvation medium was refreshed daily. Before re-addition of phosphate, the phosphate-  
153 starved, glucose-repressed cells were rapidly cooled on ice and harvested by centrifugation.  
154 The pellet was washed twice with ice-cold Mes-buffer pH 6.0 and resuspended in fresh  
155 phosphate starvation medium with 4% glucose at a cell density of 25mg cells (wet weight) per  
156 ml. After 30 min of incubation, KH<sub>2</sub>PO<sub>4</sub> was added to the culture and samples were taken at  
157 the indicated time points. For monitoring growth recovery after re-addition of phosphate, the  
158 Pi-starved cells were resuspended in fresh Pi-starvation medium supplemented with KH<sub>2</sub>PO<sub>4</sub>  
159 at an initial OD<sub>600</sub> of 0.1 and growth was followed by measuring OD<sub>600</sub> at the indicated time  
160 points. For the localization studies, cells were grown till mid-exponential phase in ScD and  
161 then starved for phosphate and/or glucose or treated with 200nM rapamycin for 6-8 hours  
162 unless indicated otherwise. All incubations were done at 30°C under continuous shaking.

163

### 164 *Sampling, extraction and determination of trehalase activity*

165 Samples of 50 mg cells (wet weight) were taken at the indicated time-points. Cells were  
166 rapidly cooled by addition of ice-cold water, centrifuged and resuspended in 500 µl of 25 mM  
167 MES buffer, 50 µM CaCl<sub>2</sub>, pH 7 for extraction. Crude cell extracts were prepared as described  
168 previously [20] and dialyzed (BRL microdialysis system) against 25 mM MES buffer pH 7  
169 with 50 µM CaCl<sub>2</sub> at 4°C. Trehalase activity in dialyzed cell extracts was determined as  
170 described previously [20]. The specific activity is expressed as nmol glucose liberated per min  
171 and per mg protein. Total amount of protein in the samples was determined using a standard  
172 method [27].

173

174

175

176

177 *Phosphate-consumption assay*

178 After addition of 1 mM  $\text{KH}_2\text{PO}_4$  to Pi-starved cells, 1 ml samples were taken and cells were  
179 rapidly pelleted at 4°C. Phosphate levels in the supernatans were determined with the Fiske-  
180 Subbarow colorimetric method for the determination of inorganic phosphate [28].

181 *RNA extraction and Northern blot analysis*

182 Samples of 120 mg cells (wet weight) were taken at the indicated time-points and rapidly  
183 cooled by addition of ice-cold water. The cells were pelleted and washed once with ice-cold  
184 water and stored at -80°C. Isolation of total RNA was performed as described previously [20].

185 Probes were labeled with  $\alpha$ -<sup>32</sup>P-dCTP using the High Prime kit (Boehringer Mannheim).  
186 Northern blots were made by separation of total RNA on gels containing 1% (w/v) agarose in  
187 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH pH 7.5 and 1% formaldehyde.  
188 Subsequently, RNA was transferred by capillary blotting to a Hybond-N membrane  
189 (Amersham) using 10× SCC buffer (1.5 M NaCl, 0.15 M Na-citrate, pH 7.0). The filters were  
190 hybridized with the <sup>32</sup>P-labelled probes consisting of fragments of the desired coding region.  
191 The blots were analyzed using PhosphorImager technology (FUJIX, BAS-1000).

192

193 *Fluorescence microscopy*

194 Cells expressing EGFP-fusion constructs were grown as described above and used directly for  
195 microscopy without fixation. Cells were viewed using a 'Leica DM4000B' fluorescence  
196 microscope. Pictures were taken with a 'Leica DFC420C' camera using the Leica Application  
197 Suite software. For FM4-64 ([N-(3-triethylammoniumpropyl)-4-(*p*-diethylamino  
198 phenylhexatrienyl) pyridinium dibromide; Molecular Probes), which specifically stains the  
199 vacuolar membrane, strains overexpressing EGFP-fused Pho87 and Pho90 were starved for 6  
200 hours for phosphate. The cells were stained with FM4-64 dye for 20 min at 4°C and incubated  
201 at RT for 60 minutes to induce transport of FM4-64 via endocytotic intermediates to the  
202 vacuolar membrane and fluorescence of both the FM4-64 and the EGFP-fusions was  
203 examined. Overlaying of images was performed with ImageJ.

204

205

206 *Reproducibility of the results*

207 All experiments were performed at least three times. For growth curves, trehalase and Pi-  
208 consumption experiments, representative figures are shown. The absolute values are  
209 somewhat variable between different experiments, but the relative differences observed  
210 between separate experiments and strains are highly reproducible. For Northern blot  
211 experiments, representative blots from three independent experiments are shown.

212

213

## 214 Results

### 215 *The deletion of PHO4 shifts rapid Pi-signaling control from Pho84 to Pho87*

216 Recent papers have demonstrated that Pho84 is the major Pi-transporter in *S. cerevisiae*  
217 involved in rapid Pi-signaling [22, 23], as the *pho84Δ* strain is unable to rapidly reverse Pi-  
218 starvation responses. However, we previously observed rapid phosphate signaling in strains  
219 deleted for *PHO4* or *PHO81* [19], both of which do not display expression of *PHO84*. Thus,  
220 either expression of *PHO84* below detection levels is sufficient to sustain phosphate signaling  
221 or another phosphate transporter takes over the function of Pho84 in the absence of Pho4  
222 activity. To investigate this in more detail, the individual Pi-transporters were deleted in a  
223 *pho4Δ* background. Upon re-addition of  $\text{KH}_2\text{PO}_4$  to Pi-starved cultures, we observed that  
224 additional deletion of *PHO4* not only restored Pi-signaling in *pho84Δ* cells, but it also shifted  
225 the Pi-signaling control towards Pho87. The latter is illustrated by the absence of trehalase  
226 activation in a *pho4Δ pho87Δ* strain (Fig. 1A), which nicely correlated with an inability to  
227 rapidly consume measurable amounts of Pi (Fig. 1B). Rapid Pi-signaling functions in part to  
228 quickly restart growth of the yeast cell, which is further highlighted by the rapid induction of  
229 ribosomal protein genes and the repression of the stress-responsive genes (Fig. S1A).  
230 Consequently, we observed that the *pho84Δ* and the *pho4Δ pho87Δ* mutant display retardation  
231 in growth resumption after addition of phosphate to Pi-starved cells when compared to the  
232 wild-type strain and the other mutants (Fig. S1B). This nicely correlates with the delayed  
233 induction of ribosomal protein genes and the delayed inhibition of stress-responsive genes in  
234 these strains (Fig. S1A). Notably, the presence of the other low-affinity Pi-transporter, i.e.  
235 Pho90, does not substitute for Pho87 when it comes to Pi-signaling in the *pho4Δ* strain (Fig.  
236 1), indicating that the two low-affinity Pi-transporters are non-redundant. Deletion of *PHO91*  
237 in a *pho4Δ* did also not affect rapid Pi-signaling under these conditions (results not shown).  
238 Pho91 was not further investigated as this low-affinity Pi-transporter was shown to mediate  
239 Pi-transport across the vacuolar membrane [6]. Together, the above described results indicate  
240 that specifically the Pho87 Pi-transporter mediates Pi-signaling in the absence of a functional  
241 Pho4 transcription factor.

242  
243 One possibility would be that Pho4 is somehow involved in transcriptional repression of  
244 *PHO87*. However, previous studies already indicated that transcription of the low-affinity Pi-  
245 transporters is independent of both Pi concentration and Pho4 activity [9]. This was further  
246 confirmed in our study, since there was no significant alteration in the expression of either  
247 *PHO87* or *PHO90* when wild-type, *pho84Δ* or *pho4Δ* cells were starved for Pi, this in contrast  
248 to the expression of *PHO84* and *PHO89*, encoding the high-affinity Pi-transport system (Fig.  
249 S1C). Altogether, these results imply that under Pi-limiting conditions, wild-type yeast cells  
250 downregulate the low-affinity Pi-transporter Pho87 and turn to the high-affinity Pho84 Pi-  
251 transporter to signal and take up the available Pi. Both processes depend on the activation of  
252 the Pho4 transcription factor, which directly induces *PHO84* expression and results in the  
253 inactivation of Pho87 at a posttranscriptional level.

254  
255 *Spl2 mediates Pi-starvation-induced vacuolar targeting of Pho87, but not of Pho90.*

256 In yeast, several plasma membrane nutrient transporters are inactivated and targeted to the  
257 vacuole in response to changes in the availability of nutrients [29-32]. To determine whether  
258 the low-affinity Pi-transporters Pho87 and Pho90 are subject to nutrient limitation-induced  
259 vacuolar targeting as well and to establish a role of Pho4 in this process, we set out to  
260 compare the intracellular localization of the EGFP-tagged Pho87 and Pho90 proteins under  
261 different nutritional conditions in the wild-type strain and in the *pho4Δ* mutant. For this, we  
262 opted to use the strains previously described [6], where chromosomal N-terminal EGFP-  
263 fusion genes of *PHO87* and *PHO90* were expressed by the TEF promoter. As shown in Fig.



264 2A, Pho87 was localized at the plasma membrane of wild-type cells grown on ScD medium,  
265 but this low-affinity transporter is endocytosed and targeted to the vacuole when cells  
266 encountered Pi-starvation. This was additionally confirmed by the deletion of *END3*,  
267 encoding an essential component for endocytosis, which prevented the internalisation of  
268 Pho87 and, in consequence, led to the stabilization of this Pi-transporter at the plasma  
269 membrane under Pi-starving conditions. Furthermore, the process could be monitored in wild-  
270 type cells when these were stained with FM4-64, a lipophilic dye that is endocytosed and used  
271 to mark endosomes and the vacuolar membrane (Fig. S2A). Intriguingly, the Pi-starvation-  
272 induced endocytosis and vacuolar targeting of Pho87 was prevented by the deletion of *PHO4*  
273 (Fig. 2A) as well as by the deletion of the Pho4-regulated gene *SPL2* (Fig. 2A and S2A). The  
274 latter was previously identified to encode a negative regulator of low-affinity Pi-transport  
275 [24]. Next, we analysed Pi-starvation-induced endocytosis and vacuolar targeting of Pho90 in  
276 the wild-type and the *pho4Δ*, *spl2Δ* and *end3Δ* mutants using the *p<sub>TEF</sub>-EGFP-PHO90*  
277 construct. In contrast to the Pho87 Pi-transporter, endocytosis of Pho90 was only prevented in  
278 the mutant strain lacking End3 and not in the strains lacking Pho4 or Spl2, indicating that the  
279 Pi-starvation-induced turnover of Pho90 occurred independent of the latter (Fig. 2B and S2B).  
280 We then examined a possible involvement of the protein kinase Rim15, another effector of the  
281 Pho85-Pho80 kinase complex [19, 33]. However, this effector did not seem to play a role in  
282 the control of the low-affinity transporters, since the deletion of *RIM15* did not affect the  
283 removal of Pho87 or Pho90 from the plasma membrane during Pi-starvation (Fig. 2A and  
284 2B).

285  
286 Recently, Spl2 was shown to interact with the N-terminal SPX-domain of Pho87 and Pho90  
287 [4]. This led us to analyse Pi-starvation-induced endocytosis and vacuolar targeting of  
288 Pho87Δ375N and Pho90Δ375N, representing Pho87 and Pho90 constructs lacking the N-  
289 terminal 375 amino acids. The endocytosis of Pho87 and Pho90 under Pi-starvation  
290 conditions was abrogated in the absence of their SPX-domain (Fig 2A and 2B). For Pho87,  
291 this observation fits with the proposed role of Spl2 as inhibitor of low-affinity Pi-transport.  
292 For Pho90, however, the Pi-starvation-induced turnover occurs independently of Spl2 and,  
293 therefore, our data suggest that this process requires the interaction of another factor with the  
294 SPX-domain.

295  
296 *The SPX-domain is essential for vacuolar targeting of Pho87 and Pho90 in response to*  
297 *glucose starvation and rapamycin treatment.*

298 Recently, it was reported that the Pi-induced degradation of Pho84 is delayed in strains with  
299 reduced PKA activity [22, 34]. As PKA is part of a nutrient-responsive network, which also  
300 comprises the protein kinases Sch9, Rim15 and TORC1, we investigated whether other  
301 nutritional cues, besides Pi, affected the internalisation and subsequent vacuolar targeting of  
302 Pho87 and Pho90. First, to test the effect of carbon source availability, cells were transferred  
303 from ScD to Sc medium lacking a C-source for 6-8 hours. Under these conditions Pho87 and  
304 Pho90 were also endocytosed and targeted to the vacuole and this process could not be  
305 prevented by deletion of *PHO4* or *SPL2* (Fig. 3A and 3B). Interestingly, shifting the cells  
306 from ScD medium to Sc containing galactose or glycerol already induced endocytosis of both  
307 low-affinity Pi-transporters (results not shown), letting us conclude that a downshift in the  
308 quality of the C-source is sufficient to initiate the process. Since Rim15 acts as a nutritional  
309 integrator in *S. cerevisiae*, we also analysed the effect of a *RIM15* deletion on endocytosis and  
310 vacuolar targeting of both Pi-transporters but, similar as for Pi-starvation, the Rim15 kinase  
311 appeared not to be required (Fig. 3A and 3B).

312

313 Next, we challenged the cells with the TORC1-inhibiting drug rapamycin, a condition known  
314 to mimic amino acid and nitrogen starvation and to induce vacuolar targeting of different  
315 permeases in yeast, such as Gap1 and Tat2 [35, 36]. As shown, treatment of yeast cells with  
316 rapamycin also triggered endocytosis and vacuolar targeting of Pho87 and Pho90 and this was  
317 again independent of Pho4, Spl2 and Rim15 (Fig. 3A and 3B). Similar results were obtained  
318 for nitrogen starvation as well (results not shown).

319  
320 Since the SPX-domain is essential for endocytosis and vacuolar targeting of Pho87 and Pho90  
321 under Pi-starvation conditions, we wondered whether this domain also directs endocytosis  
322 upon glucose starvation and rapamycin treatment. Therefore, we analysed the internalisation  
323 of the Pho87 $\Delta$ 375N and Pho90 $\Delta$ 375N constructs, which revealed that the SPX-domain is  
324 indeed required to allow endocytosis of the two low-affinity Pi-transporters under both  
325 conditions (Fig. 3A and 3B). Thus, despite the difference in the underlying control  
326 mechanisms and proteins involved, the internalization of the low-affinity Pi-transporters  
327 during Pi-starvation, glucose starvation or rapamycin treatment is based on the same  
328 principle, i.e. interactions of endocytosis-inducing proteins with the SPX-domain of the  
329 transporter.

330  
331 *The Pho90 low-affinity Pi-transporter is required for proper growth under high Pi conditions*  
332 *in the absence of a high-affinity Pi-transport system*

333 The data described above focussed on the role and turnover of the low-affinity Pi-transporters  
334 under starvation conditions. In the next section, the physiological role of the low-affinity Pi-  
335 transporters was investigated when sufficient amounts of Pi are present. Therefore, we  
336 evaluated the contribution of Pho87 and Pho90 to sustain growth of mutant strains lacking a  
337 functional high-affinity Pi-transport system, in relation to increasing levels of extracellular  
338 inorganic Pi. In these mutants, the high-affinity transport system was inactivated, either by  
339 deletion of *PHO4*, thereby inhibiting the transcription of *PHO84* and *PHO89*, or by the  
340 combined deletion of *PHO84* and *PHO89*. The absence of a high-affinity Pi-transport system  
341 results in a clear growth defect once the extracellular Pi levels drop below 250 $\mu$ M (Fig. 4A).  
342 We then additionally deleted either Pho87 or Pho90 in order to determine in more detail the  
343 contributions of each of the low-affinity Pi-transporters to sustain growth at different  
344 extracellular Pi concentrations. The *pho4* $\Delta$  *pho90* $\Delta$  and the *pho84* $\Delta$  *pho89* $\Delta$  *pho90* $\Delta$  strains  
345 already displayed a clear growth defect once the extracellular Pi concentrations dropped  
346 below 5 mM, while the *pho4* $\Delta$  *pho87* $\Delta$  and the *pho84* $\Delta$  *pho89* $\Delta$  *pho87* $\Delta$  strain displayed a  
347 growth defect only at Pi concentrations of 500 $\mu$ M and below (Fig. 4A). This indicates that  
348 mainly the Pho90 low-affinity Pi-transporter is important when sufficient amounts of  
349 extracellular Pi are available.

350  
351 These results were additionally confirmed when the growth of the different strains was further  
352 analyzed on rich YPD media and in the presence of the toxic phosphate analogue arsenate. On  
353 YPD medium, specifically the *pho4* $\Delta$  *pho90* $\Delta$  and the *pho84* $\Delta$  *pho89* $\Delta$  *pho90* $\Delta$  strains exhibit  
354 growth defects when compared to the other strains examined. Consistent with the above, these  
355 defects can be suppressed by the addition of 10mM Pi, indicating again the importance of  
356 Pho90 for Pi-uptake to sustain growth (Fig. 4B). Moreover, when arsenate was added to the  
357 medium, an increased resistance to this toxic phosphate analogue was observed for both the  
358 *pho4* $\Delta$  *pho90* $\Delta$  and the *pho84* $\Delta$  *pho89* $\Delta$  *pho90* $\Delta$  strain (Fig. 4B).

359  
360

## 361 Discussion

362 *The low-affinity Pi-transporters Pho87 and Pho90 are controlled by endocytosis in response*  
363 *to nutrient starvation*

364 Our data concerning the rapid phosphate signaling indicated that Pho4 inhibits the function of  
365 the low-affinity Pi-transporter Pho87. This inhibition appeared to be independent of *PHO87*  
366 transcription, which, in agreement with previous findings [9], is not regulated by Pi  
367 concentration or by major components of the PHO pathway, such as Pho4 and Pho84. This  
368 indicated that the regulation of Pho87 in function of Pi-availability must occur primarily at a  
369 posttranscriptional level and, consistently, we demonstrated that both the Pho87 and Pho90  
370 transporters are targeted to the vacuole when yeast cells are starved for phosphate. It was  
371 previously reported that this process was likely to involve ubiquitination-induced endocytosis  
372 [37], which is in agreement with our data showing a complete stabilization of Pho87 and  
373 Pho90 at the plasma membrane in Pi-starved *end3Δ* cells. Interestingly, the vacuolar targeting  
374 is impaired specifically for Pho87 in cells deleted for *PHO4* or *SPL2*, while Pho90 is still  
375 routed to the vacuole in the *pho4Δ* strain. As the Pho87Δ375N protein, lacking its SPX-  
376 domain, also failed to be targeted to the vacuole in the absence of phosphate, our data suggest  
377 a role for Spl2 in mediating endocytosis of Pho87 via interaction with its SPX-domain [4].  
378 Interestingly, the Pho90Δ375N protein displayed a similar localization pattern, indicating that  
379 also for Pho90, the SPX-domain is required for its endocytosis and vacuolar targeting under  
380 phosphate starvation, although in this case the process is largely independent of the Pho4/Spl2  
381 branch of the PHO-pathway. Hence, it can be concluded that two independent mechanisms  
382 regulate the selective vacuolar targeting of the Pho87 and Pho90 low-affinity phosphate  
383 transporters in response to depletion of phosphate from the medium, both of which are  
384 absolutely dependent on interactions with the N-terminal 375 amino acids of these proteins.

385  
386 Our data on endocytosis and vacuolar targeting of the low-affinity Pi-transporters clarify the  
387 results obtained in the context of phosphate signaling. Since in a wild-type strain both Pho87  
388 and Pho90 are subject to vacuolar targeting during phosphate starvation, rapid phosphate  
389 consumption and signaling is largely depending on the high-affinity transporter Pho84 (Fig.  
390 5). However, in a strain lacking Pho4, transcription of *PHO84* is absent, rendering the cells  
391 unable to signal phosphate availability via the Pho84 transporter. However, in this strain,  
392 specifically Pho87 remains stabilized at the plasma membrane under phosphate starvation,  
393 while Pho90 is still undergoing vacuolar targeting, making that rapid phosphate consumption  
394 and signaling becomes, indeed, strictly dependent on this low-affinity Pi-transporter (Fig. 5).

395  
396 We also demonstrate that besides control by Pi-availability, other nutrients are able to regulate  
397 the low-affinity Pi-transporter turnover. This was documented for glucose starvation and  
398 rapamycin-treatment, the latter being a condition that mimics starvation for nitrogen and  
399 amino acids [36]. Both conditions were found to affect both Pho87 and Pho90, and we  
400 confirm that this turnover is also driven by endocytosis [22, 37, 38]. Although the vacuolar  
401 targeting of both Pi-transporters under these conditions does not involve the Pho4/Spl2, the  
402 SPX-domains of Pho87 and Pho90 are again essential for this process. This strengthens our  
403 hypothesis that other SPX-binding factors are involved in mediating endocytosis and vacuolar  
404 targeting. Furthermore, the data indicate that both glucose starvation and rapamycin treatment  
405 induces a general vacuolar sorting response that overrules the Pi-specific feedback loops. This  
406 may allow the cell to adjust Pi-uptake not only as a function of the external Pi concentration,  
407 but also to balance the Pi-uptake in respect to other metabolic processes, thereby preventing  
408 an overflow of internal Pi-pools.

409

410 As our study indicates that both Spl2 and the SPX-domain of Pho87 are required for Pi-  
411 starvation-induced endocytosis of Pho87, these results fit nicely with the previously reported  
412 interaction of Spl2 with the SPX-domain of this Pi-transporter [4]. In the same study,  
413 however, a similar interaction of Spl2 was observed with the SPX-domain of Pho90, while we  
414 did not observe a necessity for Spl2 in the endocytosis of Pho90 upon Pi-starvation, although  
415 the SPX-domain of the latter still seemed absolutely required. These results seem  
416 contradictory, but one has to keep in mind the differences in experimental approaches in our  
417 study and the previous one. The negative interaction between Spl2 and Pho90, as described by  
418 [4], was seen in strains overexpressing *PHO90* and/or *SPL2* on rich YPD medium, which  
419 contains intermediate levels of Pi. In contrast, effects in our study were observed upon  
420 deletion of *SPL2* in Pi-starvation medium. In addition, our results obtained under different  
421 nutritional conditions, as described above, indicate that other factors, besides Spl2, exist that  
422 mediate endocytosis of both Pho90 and Pho87. Consequently, overexpression of *SPL2* may  
423 generate dominant effects overruling the regulation of the Pi-transporters by other factors,  
424 while deletion of *SPL2* makes the regulation of Pho87 and Pho90 exclusively dependent on  
425 these factors. It is therefore very likely that Spl2 mediates the regulation of Pho90 activity  
426 under certain physiological conditions not addressed in this study. In this respect, we note that  
427 although a transcriptional regulation of *SPL2* in response to Pi-levels is already documented  
428 [10, 24], other modes of regulation of Spl2 function may exist which respond to different  
429 environmental cues.

430

#### 431 *Non-redundant functions for the Pho87 and Pho90 low-affinity Pi-transporters*

432 In this study, we unveiled different situations in which either the Pho87 or the Pho90 Pi-  
433 transporter is required for signaling and/or optimal growth. Indeed, we showed that in a  
434 *pho4Δ* background, phosphate signaling and rapid growth recovery from Pi-starvation  
435 depends specifically on the Pho87 transporter, while under conditions where sufficient  
436 amount of Pi are available, Pho90 is the most important low-affinity Pi-transporter. These  
437 transporter-specific requirements signify that Pho87 and Pho90 represent non-redundant Pi-  
438 transporters which are tuned by the integration of multiple nutrient signaling mechanisms in  
439 order to adjust Pi-transport capacity to the general nutritional status of the environment.

440

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447

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- 554
- 555
- 556



557 **Table 1: Strains used in this study**

558	<b>Strain</b>	<b>Genotype</b>	<b>Reference</b>
559	WT (BY4741)	Mata <i>his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i>	Y.K.O. (Invitrogen)
560	<i>pho84Δ</i>	<i>pho84::KANMX4</i>	Y.K.O. (Invitrogen)
561	<i>pho4Δ</i>	<i>pho4::KANMX4</i>	Y.K.O. (Invitrogen)
562	<i>pho4Δ pho84Δ</i>	<i>pho4::KANMX4 pho84::KANMX4</i>	This study
563	<i>pho4Δ pho87Δ</i>	<i>pho4::KANMX4 pho87::KANMX4</i>	This study
564	<i>pho4Δ pho90Δ</i>	<i>pho4::KANMX4 pho90::KANMX4</i>	This study
565	<i>pho84Δ pho89Δ</i>	<i>pho84::KANMX4 pho89::LEU2</i>	This study
566	<i>pho84Δ pho89Δ pho90Δ</i>	<i>pho84::KANMX4 pho89::LEU2</i>	
567		<i>pho90::KANMX4</i>	This study
568	<i>pho84Δ pho87Δ pho89Δ</i>	<i>pho84::KANMX4 pho87::KANMX4</i>	
569		<i>pho89::LEU2</i>	This study
570	$P_{TEF}$ -EGFP- <i>PHO87</i>	Mata NatMx- $P_{TEF}$ -EGFP- <i>PHO87</i>	Hürlimann <i>et al.</i> , 2007
571	$P_{TEF}$ -EGFP- <i>PHO87Δ375N</i>	Mata NatMx- $P_{TEF}$ -EGFP- <i>PHO87Δ375N</i>	Hürlimann <i>et al.</i> , 2009
572	<i>pho4Δ</i> $P_{TEF}$ -EGFP- <i>PHO87</i>	<i>pho4::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO87</i>	This study
573	<i>spl2Δ</i> $P_{TEF}$ -EGFP- <i>PHO87</i>	<i>spl2::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO87</i>	This study
574	<i>end3Δ</i> $P_{TEF}$ -EGFP- <i>PHO87</i>	<i>end3::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO87</i>	This study
575	<i>rim15Δ</i> $P_{TEF}$ -EGFP- <i>PHO87</i>	<i>rim15::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO87</i>	This study
576	$P_{TEF}$ -EGFP- <i>PHO90</i>	Mata NatMx- $P_{TEF}$ -EGFP- <i>PHO90</i>	Hürlimann <i>et al.</i> , 2007
577	$P_{TEF}$ -EGFP- <i>PHO90Δ375N</i>	Mata NatMx- $P_{TEF}$ -EGFP- <i>PHO90Δ375N</i>	Hürlimann <i>et al.</i> , 2009
578	<i>pho4Δ</i> $P_{TEF}$ -EGFP- <i>PHO90</i>	<i>pho4::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO90</i>	This study
579	<i>spl2Δ</i> $P_{TEF}$ -EGFP- <i>PHO90</i>	<i>spl2::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO90</i>	This study
580	<i>rim15Δ</i> $P_{TEF}$ -EGFP- <i>PHO90</i>	<i>rim15::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO90</i>	This study
581	<i>end3Δ</i> $P_{TEF}$ -EGFP- <i>PHO90</i>	<i>end3::KANMX4</i> NatMx- $P_{TEF}$ -EGFP- <i>PHO90</i>	This study

582 **Figure legends**583 **Figure 1: The low-affinity Pi-transporter Pho87 is essential for rapid Pi-signaling in cells**  
584 **lacking the transcriptional regulator Pho4**

585 The wild-type (WT) strain ( $\square$ ) and the mutants *pho84 $\Delta$*  ( $\triangle$ ), *pho4 $\Delta$*  ( $\blacktriangle$ ), *pho4 $\Delta$  pho84 $\Delta$*  ( $\blacklozenge$ ),  
586 *pho4 $\Delta$  pho87 $\Delta$*  ( $\blacksquare$ ) and *pho4 $\Delta$  pho90 $\Delta$*  ( $\diamond$ ) are grown to mid-exponential phase in ScD  
587 medium after which they are transferred to Pi-starvation medium for 3 days. The cells were  
588 then analysed for (A) activation of trehalase after addition of 10 mM  $\text{KH}_2\text{PO}_4$  and (B) Pi  
589 consumption after re-addition of 1 mM  $\text{KH}_2\text{PO}_4$  to the Pi-starved cells.

590

591 **Figure 2: Spl2 mediates the vacuolar targeting of Pho87 but not of Pho90 under Pi-**  
592 **starving conditions**

593 Cells carrying a chromosomal  $P_{\text{TEF}}$ -EGFP-*PHO87* (A),  $P_{\text{TEF}}$ -EGFP-*PHO87 $\Delta$ 375N* (A),  $P_{\text{TEF}}$ -  
594 EGFP-*PHO90* (B) or a  $P_{\text{TEF}}$ -EGFP-*PHO90 $\Delta$ 375N* (B) construct were used to monitor the  
595 localization of the low-affinity transporters. Cells were grown exponentially (Exp) and  
596 transferred for 6-8 hours to Pi-starvation medium (-Pi). As indicated, the wild-type cells or  
597 mutants carrying a *PHO4*, *SPL2*, *RIM15* or *END3* deletion were used. Over more than 300  
598 fluorescent cells were counted and >90% of the cells exhibit the phenotypes as visualised in  
599 the picture.

600

601 **Figure 3: The SPX-domain of Pho87 and Pho90 mediates the vacuolar targeting of both**  
602 **Pi-transporters during glucose starvation conditions and after rapamycin treatment**

603 Cells carrying a chromosomal  $P_{\text{TEF}}$ -EGFP-*PHO87* (A),  $P_{\text{TEF}}$ -EGFP-*PHO87 $\Delta$ 375N* (A),  $P_{\text{TEF}}$ -  
604 EGFP-*PHO90* (B) or a  $P_{\text{TEF}}$ -EGFP-*PHO90 $\Delta$ 375N* (B) construct were used to monitor the  
605 localization of the low-affinity transporters. Cells were grown exponentially (Exp) and  
606 transferred for 6-8 hours to medium without glucose (-D). Also, the localization of both Pi-  
607 transporters was monitored after treatment of exponentially grown cells for 6-8 hours with  
608 200nM rapamycin (Rap). As indicated, the wild-type cells or mutants carrying a *PHO4*, *SPL2*,  
609 *RIM15* or *END3* deletion were used. Over more than 300 fluorescent cells were counted and  
610 >90% of the cells exhibit the phenotypes as visualised in the picture.

611

612 **Figure 4: The Pho90 Pi-transporter is required under high Pi conditions in absence of a**  
613 **high-affinity Pi-transport system**

614 (A) To evaluate the contribution of the Pho87 and the Pho90 low-affinity Pi-transporters to  
615 strains lacking a functional high-affinity Pi-transport system, the growth of strains containing  
616 only Pho87 (the *pho4 $\Delta$  pho90 $\Delta$*  and the *pho84 $\Delta$  pho89 $\Delta$  pho90 $\Delta$*  strain) or Pho90 (the *pho4 $\Delta$*   
617 *pho87 $\Delta$*  and *pho84 $\Delta$  pho89 $\Delta$  pho87 $\Delta$*  strain) at the plasma membrane, was compared with the  
618 growth of strains only containing both low-affinity Pi-transporters (the *pho4 $\Delta$*  and the *pho84 $\Delta$*   
619 *pho89 $\Delta$*  strain). Cells were grown in ScD medium and tenfold serial dilutions were spotted on  
620 Pi-starvation medium containing a specific amount of inorganic phosphate. Pictures were  
621 taken after 2-3 days. (B) To evaluate the growth of these strains in the presence of the toxic  
622 phosphate analogue arsenate, cells were grown in YPD medium and tenfold serial dilutions  
623 were spotted on YPD, YPD containing an additional 10mM inorganic phosphate and/or 5mM  
624  $\text{AsO}_4$ . Pictures were taken after 3-5 days.

625

626 **Figure 5: Regulation of yeast Pi-transporters during phosphate starvation**

627 When Pi becomes limiting, the activity of the Pho85-Pho80 kinase is inhibited by Pho81,  
628 leading to nuclear translocation of the transcription factor Pho4. In the nucleus, Pho4 activates  
629 expression of typical PHO genes such as *PHO84* and *SPL2*. Induction of Spl2 activity  
630 stimulates vacuolar targeting of Pho87, while Pho90 is targeted to the vacuole in a Spl2-  
631 independent manner. Although not depicted here, we also demonstrated that under acute

632 glucose starvation and after the addition of rapamycin, the low-affinity Pi-transporters Pho87  
633 and Pho90 are both targeted to the vacuole in a Spl2-independent way.

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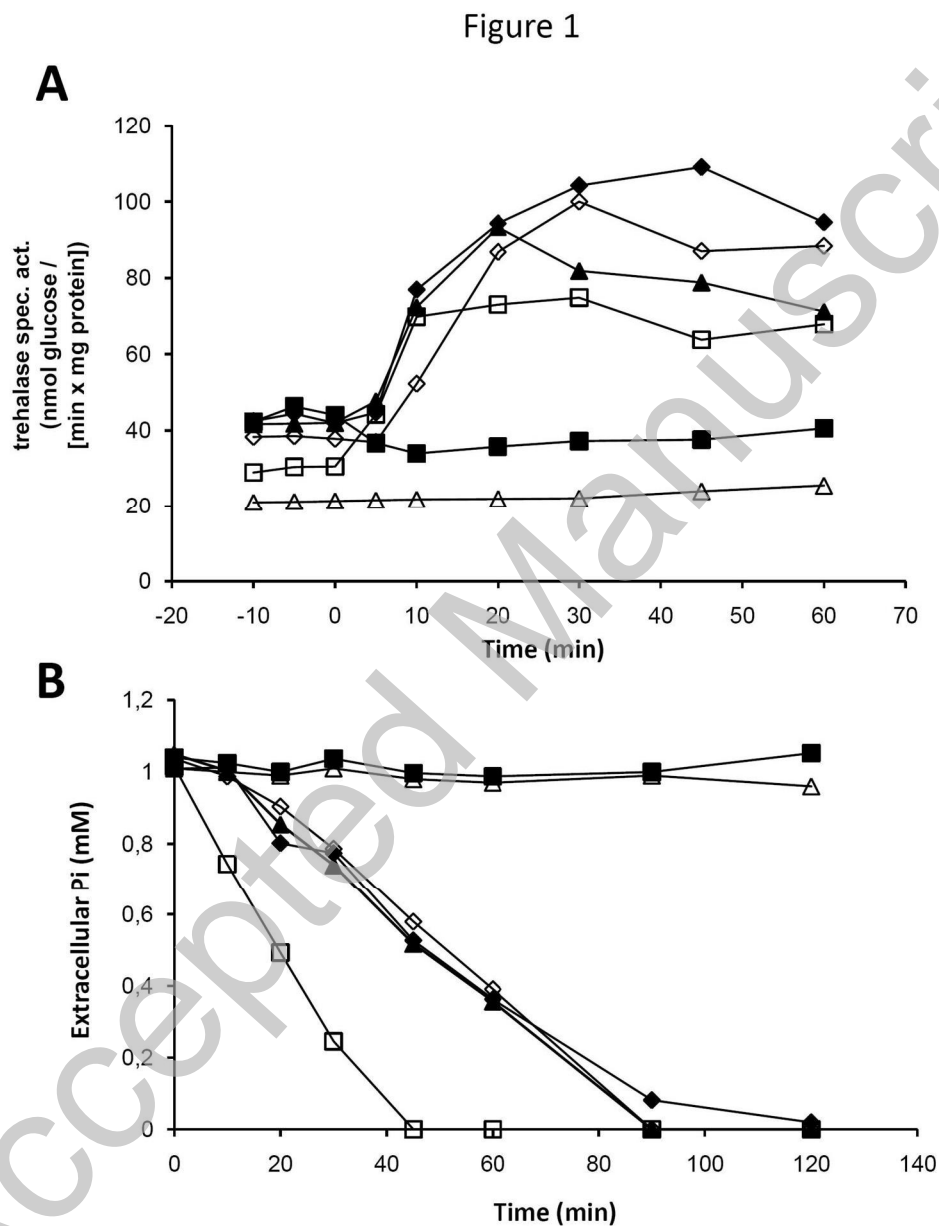


Figure 2

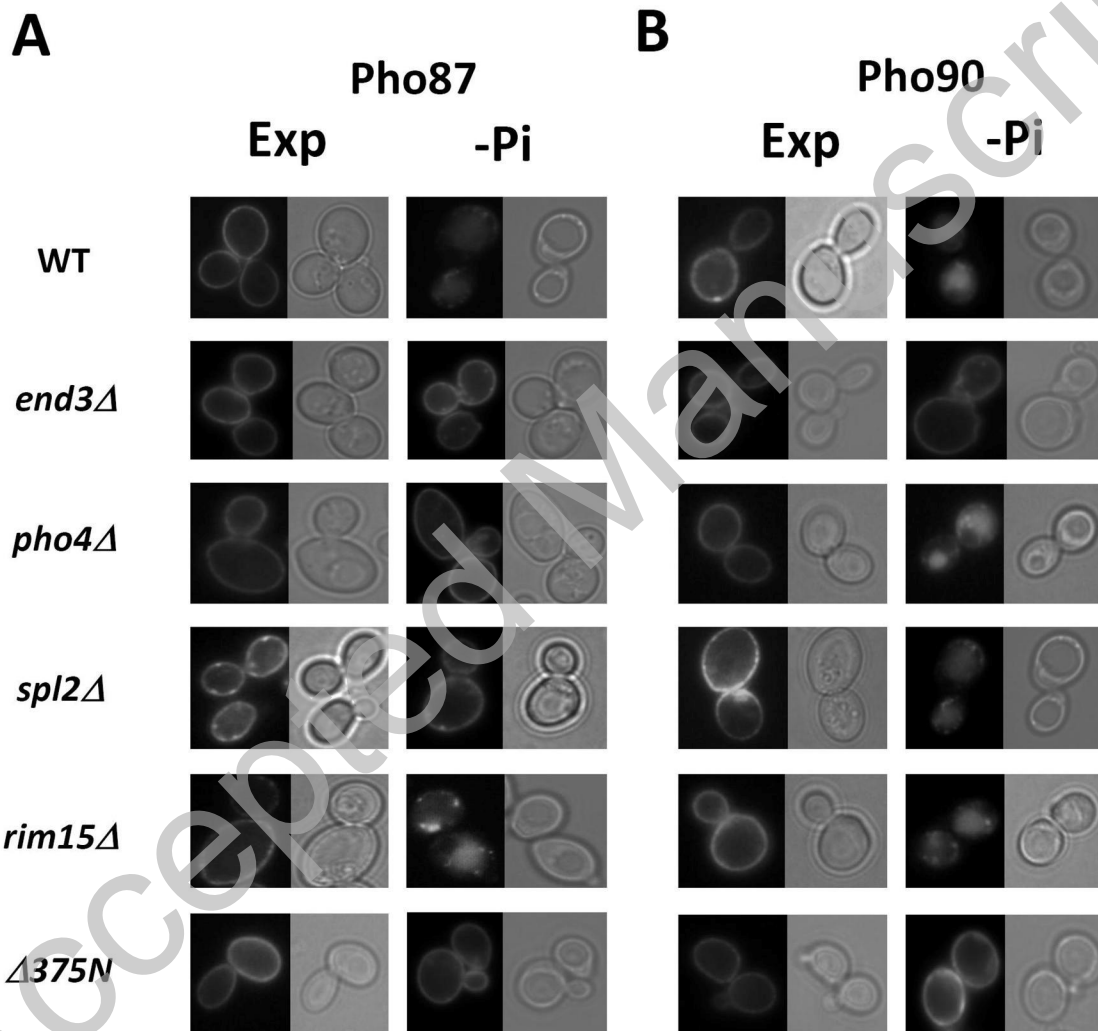


Figure 3

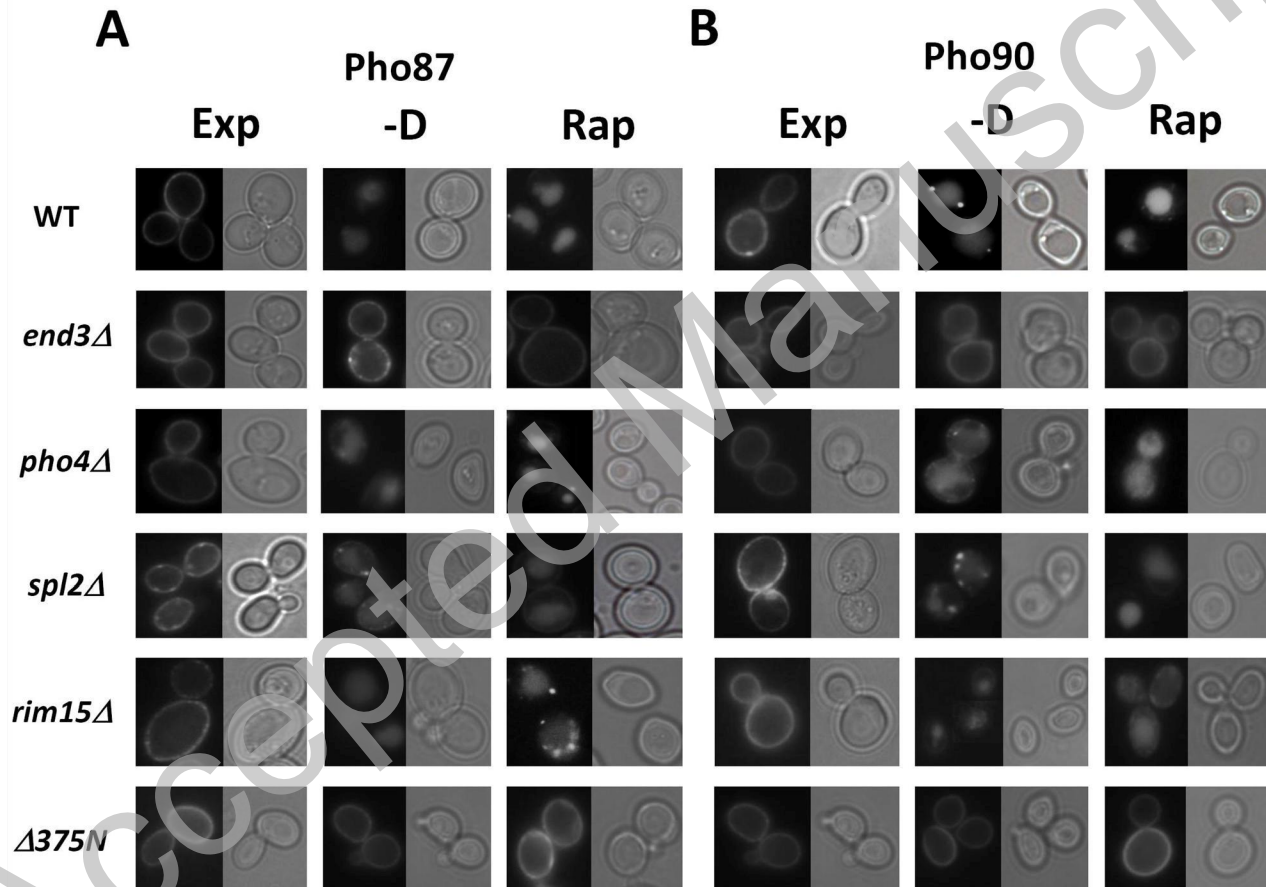
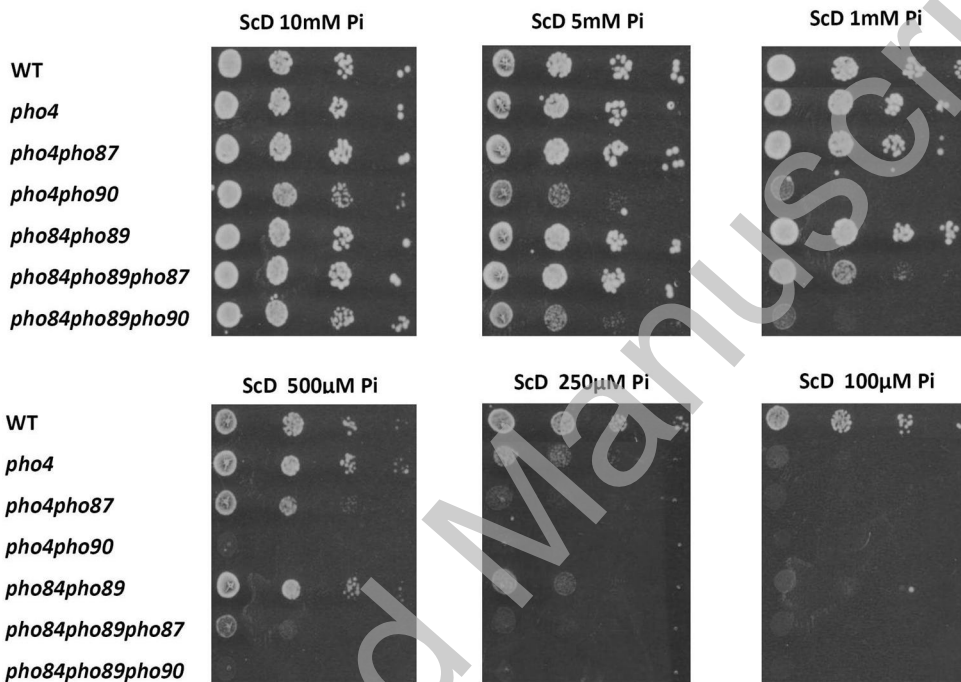


Figure 4

A



B

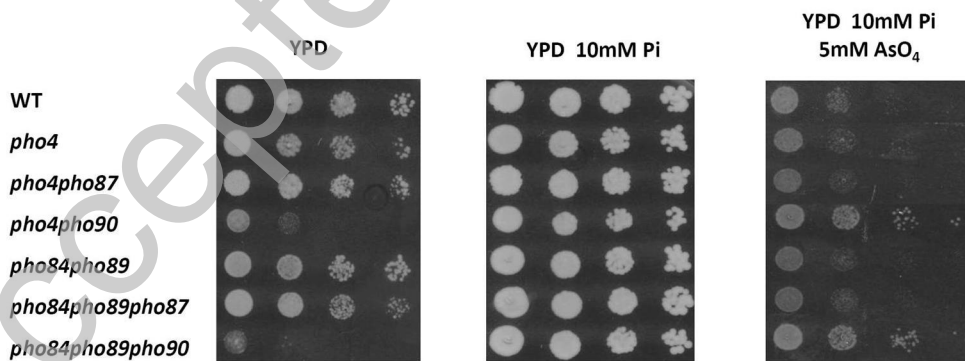


Figure 5

