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Pho91 Is a Vacuolar Phosphate Transporter That Regulates Phosphate and Polyphosphate Metabolism in *Saccharomyces cerevisiae*

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Inorganic polyphosphate (poly P) is a biopolymer that occurs in all organisms and cells and in many cellular compartments. It is involved in numerous biological phenomena and functions in cellular processes in all organisms. However, even the most fundamental aspects of poly P metabolism are largely unknown. In yeast, large amounts of poly P accumulate in the vacuole during growth. It is neither known how this poly P pool is synthesized nor how it is remobilized from the vacuole to replenish the cytosolic phosphate pool. Here, we report a systematic analysis of the yeast phosphate transporters and their function in poly P metabolism. By using poly P content as a read-out, it was possible to define novel functions of the five phosphate transporters: Pho84, Pho87, Pho89, Pho90, and Pho91, in budding yeast. Most notably, it was found that the low-affinity transporter Pho91 limits poly P accumulation in a strain lacking *PHO85*. This phenotype was not caused by a regulatory effect on the *PHO* pathway, but can be attributed to the unexpected localization of Pho91 in the vacuolar membrane. This finding is consistent with the hypothesis that Pho91 serves as a vacuolar phosphate transporter that exports phosphate from the vacuolar lumen to the cytosol.

INTRODUCTION

Phosphate is an essential macronutrient: it is an important component of nucleic acids and phospholipids, represents a source of energy in nucleotides, modulates protein activities, or serves as a signal through phosphorylation of specific amino acid residues, and it can be polymerized to form inorganic polyphosphate (poly P). But even though yeast can store up to 20% of its dry weight as poly P (Kornberg *et al.,* 1999) and furthermore, many pathways are known to be involved in poly P metabolism (Freimoser *et al.,* 2006), it is still not known how this polymer is synthesized in yeast or any higher eukaryote. To learn more about the regulation of the metabolism of phosphate and poly P, we performed a detailed study of the yeast phosphate transporters, the *PHO* pathway, and poly P levels.

The yeast genome encodes five phosphate transporters (Pho84, Pho87, Pho89, Pho90, and Pho91) that are involved in the intricately regulated phosphate uptake (Wykoff and O'Shea, 2001). Pho84 and Pho89 are two high-affinity transporters that are both regulated by the *PHO* pathway (Bun-Ya *et al.,* 1991; Martinez and Persson, 1998): Under low-phosphate conditions, the transcription factor Pho4 is dephosphorylated and localized in the nucleus, where it activates transcription of phosphate-regulated genes such as *PHO84* and *PHO89* (Lenburg and O'Shea, 1996; O'Neill *et al.,* 1996; Springer *et al.,* 2003). In the high-phosphate state, Pho4 is phosphorylated by the cyclin-dependent kinase Pho85, which causes its relocalization to the cytosol and concomitant down-regulation of phosphate responsive genes (Lenburg and O'Shea, 1996; O'Neill *et al.,* 1996; Springer *et al.,* 2003). Despite its strict regulation by the *PHO* pathway, Pho84 is the most important phosphate transporter in yeast, even in high-phosphate media (Wykoff and O'Shea, 2001). Transcription of *PHO84* and *PHO89* is not only regulated by the *PHO* pathway, but for example, also by the SAGA and the SWI-SNF complexes (Lee *et al.,* 2000; Sudarsanam *et al.,* 2000; Bhaumik and Green, 2002) or by shifts to acidic or alkaline pH, the calcineurine pathway, or the cell cycle (Causton *et al.,* 2001; Serrano *et al.,* 2002; Ruiz *et al.,* 2003; Luan and Li, 2004). In the absence of the high-affinity transporters, the low-affinity transporters, Pho87, Pho90, and Pho91, are necessary for phosphate transport, but otherwise exhibit few phenotypes if deleted (Wykoff and O'Shea, 2001). In addition to phosphate uptake, the three low-affinity phosphate transporters are involved in the external sensing of phosphate and the regulation of the phosphate-signaling pathway (Auesukaree *et al.,* 2003; Giots *et al.,* 2003; Pinson *et al.,* 2004).

The *PHO* pathway has been characterized in great detail by studying secreted acid phosphatase (rAPase) activity, *PHO84* transcription, and Pho84 localization, or trehalase activity (Petersson *et al.,* 1999; Auesukaree *et al.,* 2003, 2004; Giots *et al.,* 2003; Pinson *et al.,* 2004; Huang and O'Shea, 2005) and because of its function as a phosphate store, poly P was specifically studied as a potential regulator (Neef and Kladde, 2003; Auesukaree *et al.,* 2004; Thomas and O'Shea, 2005). But the regulation of poly P metabolism by the *PHO* pathway and by phosphate transporters has not been studied systematically. Here, we used the poly P content as the read-out for a thorough characterization of mutant strains affected in the five phosphate transporters. With poly P content

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Abbreviations used: GFP, green fluorescent protein; HA, hemagglutinin; poly P, inorganic polyphosphate; rAPase, acid phosphatase; SC medium, synthetic complete medium; YPD, yeast peptone dextrose.

as a distinguishing mark, it was possible to define novel functions of the low-affinity phosphate transporters in phosphate, poly P, and general cell metabolism. Most notably, we suggest that Pho91 is an intracellular phosphate transporter that exports phosphate from the vacuole and thereby regulates intracellular phosphate homeostasis and poly P levels.

MATERIALS AND METHODS

Yeast Strains

All yeast strains used in this study are based on the haploid knock-out (YKO) strains from the *Saccharomyces* Genome Deletion Project (Winzeler *et al.*, 1999), which were generated in the background of the strain BY4741 (MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*; Brachmann *et al.*, 1998). A detailed listing of all strains used and created in this study is given in Table 1.

Cultivation and Handling of Yeast Strains

Strains were routinely grown at 30°C in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), with the optional addition of 200 mg/l G418 (PAA Laboratories Gmbh, Pasching, Austria), 300 mg/l hygromycin (Apollo Scientific, Bredbury, United Kingdom) or 100 mg/l clonNat (Werner BioAgents, Jena, Germany). Low-orthophosphate YPD medium (YPD-Pi) was prepared by precipitating free phosphate with ammonium as described previously (Kaneko *et al.*, 1982; Werner *et al.*, 2005). The strains EY57 and EY916-EY922 were a gift from Dr. E. O'Shea (Harvard University) and Dr. D. Wykoff (Villanova University) and were maintained on YPGal medium (YPD medium containing 20 g/l galactose instead of glucose; Wykoff and O'Shea, 2001). Strains harboring plasmids were kept on SC medium lacking the corresponding amino acid (0.68 g/l yeast nitrogen base, complete supplement dropout mix [both either from Qbiogene, Carlsbad, CA, or Formedium, Norfolk, United Kingdom], 20 g/l glucose). For poly P analysis, strains were always precultured for 3 d, and fresh medium was inoculated to an OD_{600} = 1. Cells were harvested at different time points, dissolved in 1 M H_2SO_4, and stored at −20°C until extraction.

Manipulation of Yeast Strains

Plasmids were transformed into yeast by the method of Gietz *et al.* (1992). The kanamycin marker in the YKO strains was replaced with either the nourseothricine (natMX4, clonNat) or the hygromycin (hphMX4) resistance gene by transformation with Not I-digested plasmids pAG25 (natMX4) or pAG32

Table 1. Yeast strains that were used or created in the course of this study

Strain	Genotype	Reference
EY57	*MAT**a** ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*	Schwob and Nasmyth (1993); Wykoff and O'Shea (2001)
EY916	*MAT**a** pho84Δ::HIS3 pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ::KlURA3*	Wykoff and O'Shea (2001)
EY917	*MAT**a** pho84Δ::HIS3 pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ ADE2*	Wykoff and O'Shea (2001)
EY918	*MAT**a** pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ::KlURA3 ADE2*	Wykoff and O'Shea (2001)
EY919	*MAT**a** pho84Δ::HIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ::KlURA3 ADE2*	Wykoff and O'Shea (2001)
EY920	*MAT**a** pho84Δ::HIS3 pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho91Δ::KlURA3 ADE2*	Wykoff and O'Shea (2001)
EY921	*MAT**a** pho84Δ::HIS3 pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 ADE2*	Wykoff and O'Shea (2001)
EY922	*MAT**a** pho84Δ::HIS3 pho87Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ::KlURA3 ADE2*	Wykoff and O'Shea (2001)
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al.* (1998)
*pho84*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho84Δ::kanMX4*	Winzeler *et al.* (1999)
*pho85*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::kanMX4*	Winzeler *et al.* (1999)
*pho87*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho87Δ::kanMX4*	Winzeler *et al.* (1999)
*pho89*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho89Δ::kanMX4*	Winzeler *et al.* (1999)
*pho90*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho90Δ::kanMX4*	Winzeler *et al.* (1999)
*pho91*Δ	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho91Δ::kanMX4*	Winzeler *et al.* (1999)
FFSc228 (*pho85*ΔNAT)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4*	This study
FFSc370 (*pho85*Δ *pho84*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 pho84Δ::kanMX4*	This study
FFSc256 (*pho85*Δ *pho87*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 pho87Δ::kanMX4*	This study
FFSc271 (*pho85*Δ *pho89*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 pho89Δ::kanMX4*	This study
FFSc269 (*pho85*Δ *pho90*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 pho90Δ::kanMX4*	This study
FFSc266 (*pho85*Δ *pho91*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 pho91Δ::kanMX4*	This study
FFSc519 (P_{TEF} GFP-*PHO90*)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 natNT2-P_{TEF}-yeGFP-PHO90*	This study
FFSc486 (P_{TEF} GFP-*PHO91*)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 natNT2-P_{TEF}-yeGFP-PHO91*	This study
FFSc501 (P_{TEF} GFP-*PHO91 pho85*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::kanMX4 natNT2-P_{TEF}-yeGFP-PHO91*	This study
FFSc528 (P_{TEF} *PHO91*)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kanMX4-P_{TEF}-PHO91*	This study
FFSc634 (P_{CYC1} GFP-*PHO91*)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 natNT2-P_{CYC1}-yeGFP-PHO91*	This study
FFSc482 (P_{ADH} GFP-*PHO91*)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 natNT2-P_{ADH1}-yeGFP-PHO91*	This study
FFSc528 (P_{TEF} *PHO91 pho85*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho85Δ::natMX4 kanMX4-P_{TEF}-PHO91*	This study
FFSc412 (*PHO84*-GFP)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PHO84-yeGFP:HIS3MX6*	This study
FFSc609 (*PHO84*-GFP *pho87*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho87Δ::kanMX4 PHO84-yeGFP:HIS3MX6*	This study
FFSc610 (*PHO84*-GFP *pho90*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho90Δ::kanMX4 PHO84-yeGFP:HIS3MX6*	This study
FFSc611 (*PHO84*-GFP *pho91*Δ)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho91Δ::kanMX4 PHO84-yeGFP:HIS3MX6*	This study
FFSc562 (P_{TEF}-*PHO87*-GFP)	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 natNT2- P_{TEF}-yeGFP-PHO87*	This study

The "EY-strains" were derived from the strain EY57, which corresponds to the strain K699 (Schwob and Nasmyth, 1993).

(hphMX4; Goldstein and McCusker, 1999), respectively. To inactivate two genes, these markers were amplified with flanking up- and downstream genomic sequences (with the "A" and "D" primers). Double deletion strains were created by transforming existing single YKO strains with these PCR products following the high-efficiency yeast transformation protocol (Gietz and Woods, 2002). Some double deletions were also created by crossing a bait strain to selected single YKO strains (Tong *et al.*, 2001) or by crossing and tetrad dissection according to standard procedures (Kaiser *et al.*, 1994). All strains showed almost identical phenotypes irrespective of the method used for their generation. All double deletion strains were verified by PCR with the "A," "B," and kanB primers. The yeast phosphate transporters Pho87, Pho90, and Pho91 were endogenously tagged with green fluorescent protein (GFP) at the N-terminus by the PCR-based epitope-tagging strategy (Janke *et al.*, 2004). The sequences encoding the GFP and/or the different promoters were amplified with the S1 and S4 primer pair from the plasmids pYM-N9, pYM-N13, pYM-N17, pYM-N18, and pYM-N21 (for the ADH, CYC1, GPD, and TEF promoters, respectively; Janke *et al.*, 2004). In the resulting strains the endogenous promoter is replaced with a ADH, CYC1, GPD, or TEF promoter, and the GFP tag is fused to the N-terminus of the Pho87, Pho90, or Pho91. The correct fusion of the N-terminal GFP with Pho91 was also verified by sequencing the region of the junction of these two parts. Pho84 was tagged by integrating a PCR fragment of the GFP tag and a HIS-selection marker (amplified with the S2 and S3 primers from the plasmid pYM28) at the C-terminus.

DNA Manipulations

Yeast genomic DNA was isolated following the smash-and-grab protocol (Rose *et al.*, 1990). Modified versions of the plasmids pRS416-ADH, pRS416-TEF, and pRS426-GPD (Mumberg *et al.*, 1995) were made by the introduction of the sequence for one hemagglutinin (HA) tag. The novel plasmids (pRS416-ADH-HA, pRS416-TEF-HA, and pRS426-GPD-HA) allow C-terminal HA tag fusions by cloning using the XmaI restriction site. The gene encoding *PHO84* was cloned into the above mentioned vectors with the restriction sites EcoRI and XmaI that were included in the primers used for amplification. The insert in the resulting construct (pRS416-ADH-HA-*PHO84*, pRS416-TEF-HA-*PHO84*, and pRS426-GPD-HA-*PHO84*) were verified by sequencing.

Quantification of Poly P and Total Phosphate Content

Poly P was extracted, purified, and quantified as previously described (Werner *et al.*, 2005). In short, 1 OD_{600} equivalent of cells was harvested and pelleted. The supernatant was removed, 50 μl 1 M H_2SO_4 was added, and the suspension was neutralized with 50 μl 2 M NaOH and 100 μl Tris-malate buffer (1 M Tris, 0.5 M malate, pH 7.5, 6% neutral red solution [0.1% neutral red in 70% ethanol]). Cell fragments were removed by centrifugation. After addition of 600 μl 6 M NaI, the extracts were applied to Qiagen PCR purification columns (Chatsworth, CA). The columns were washed twice with wash buffer (10 mM Tris buffer, pH 7.5, 50% ethanol, 1 mM EDTA, and 100 mM NaCl). Poly P was eluted in 100 μl H_2O, and 50 μl of this eluate was specifically digested with *Saccharomyces cerevisiae* exopolyphosphatase. To quantify the released phosphate, 86 μl of 28 mM ammonium heptamolybdate (in 2.1 M H_2SO_4) and 64 μl of 1.6 mM malachite green (in 0.35% polyvinyl alcohol) were added. The malachite green solution was measured in a BioTek PowerWaveTM XS microplate spectrophotometer (BioTek Instruments, Winooski, VT) at 600 nm and compared with that of phosphate standards (5–100 μM Pi; Cogan *et al.*, 1999). To quantify total phosphate content, 0.5 OD_{600} equivalent of cells were pelleted, resuspended in 200 μl 1 M H_2SO_4, and heated in a boiling water bath for 20 min. Released phosphate was quantified as described above.

Acid Phosphatase Assay

Acid Phosphatase (rAPase) activity was assayed according to Huang and O'Shea (2005) in 50 μl cell suspension by adding 200 μl *p*-nitrophenyl-phosphate (20 mM in 100 mM sodium acetate, pH 4.2). After 5–15 min reaction time at 28°C, 200 μl of 10% ice-cold trichloroacetic acid and 400 μl sodium carbonate solution (2 M) were added, and the OD_{420} was measured. The measurements were normalized with the cell density (OD_{600}), the reaction time, and the sample volume (in ml). rAPase activity (Miller units) was obtained by multiplying the normalized OD_{420} values with a factor of 1000.

β-Galactosidase Reporter Assay

The *PHO84*-lacZ and the *ACT1*-lacZ reporter plasmids have been described earlier (Hughes *et al.*, 2001) and were a gift from Dr. S. Fields (University of Washington). The β-galactosidase reporter assay was performed similar to the protocols of Möckli and Auerbach (2004) and Miller (1972). To 10 μl of a yeast culture, 500 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7), 10 μl $CHCl_3$, and 15 μl SDS (0.2%) were added. The cells were briefly vortexed and incubated for 5 min at 28°C, and the reaction was started by the addition of 100 μl ONPG solution (4 mg/ml *O*-nitrophenyl β-D-galactopyranoside in Z-buffer). As soon as the solution turned yellow, the reaction was stopped by the addition of 250 μl of 2 M Na_2CO_3 solution. The cells were spun down, and absorption in the supernatant was measured at 420 nm. The values were normalized with the reaction time, the cell density (OD_{600}), and the sample volume (in ml). LacZ activity (Miller units) was calculated by multiplication with 1000. For each strain three independent transformants were measured, and all experiments were performed at least twice.

FM4-64 Staining and Confocal Microscopy

Vacuoles were stained with FM4-64 as described (Vida and Emr, 1995). In short, yeast cultures were grown in YPD or YPD-Pi to an $OD_{600} \approx 0.5$–2, and 3 OD_{600} units were concentrated in 100 μl YPD (or YPD-Pi) and stained at 30°C for 15–20 min with 60 μM FM4-64 (8 mM stock solution in DMSO). Cells were pelleted (700 × *g*, 3 min), resuspended in 5 ml YPD (or YPD-Pi), and shaken at 30°C for 45–60 min, and then the cells were washed three times with 1 ml PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 8.7 mM NaCl, pH 7.3) or TBS (50 mM Tris, 150 mM NaCl, pH 7.5, for cells grown in YPD-Pi). Cells were then applied to agarose-coated microscope slides (Hailey *et al.*, 2002) and observed with a confocal laser scanning microscope (Leica DM IRBE and Leica TCS SP laser; Leica, Unterentfelden, Switzerland) using an ArKr laser at 488 and 568 nm for excitation. Pictures of the stained samples and their controls were not processed digitally except for overlay of different channels and contrast adjustments.

RESULTS

Pho84 Is the Most Important Phosphate Transporter for poly P Metabolism

First, we quantified poly P and total phosphate in strains lacking either of the five phosphate transporters: Pho84, Pho87, Pho89, Pho90, or Pho91. Poly P levels were most strongly affected by the deletion of *PHO84* and *PHO87* and less so by the inactivation of *PHO89* (Figure 1). The total phosphate content was reduced to a lesser extent in these three strains (Figure 1). In contrast, strains lacking either Pho90 or Pho91 did not have strongly reduced poly P levels, and the *pho91*Δ strain even showed a slight but not significant increase in poly P (Figure 1). To further characterize the effect of each phosphate transporter on poly P levels in yeast, the mutant strains expressing only one of the five transporters (Wykoff and O'Shea, 2001) were tested. The strain that contains Pho84 but lacks all other phosphate transporters (EY918) had poly P levels similar to the wild-type strain (EY57; Figure 2). However, none of the other phosphate transporters could support measurable amounts of poly P by itself (Figure 2). This suggested that Pho84 is the most important transporter for the maintenance of normal poly P levels.

The PHO Pathway Affects poly P Levels by Regulating PHO84, and Low-Affinity Phosphate Transporters Limit poly P Accumulation in a pho85Δ Strain

It has been shown previously that inactivation of *PHO85* causes hyper-accumulation of poly P (McDonald *et al.*, 2001).

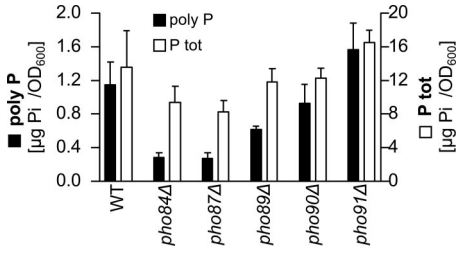

Figure 1. Poly P (■, left *y*-axis) and total phosphate (P tot, □, right *y*-axis) levels in single deletion strains of the five yeast phosphate transporters. The six strains were grown in YPD medium, and samples were taken at different time points. The figure shows only the measurements of the samples harvested after 4 h (late exponential phase). The bars represent the mean of four poly P extractions and quantifications, and the SD is indicated.

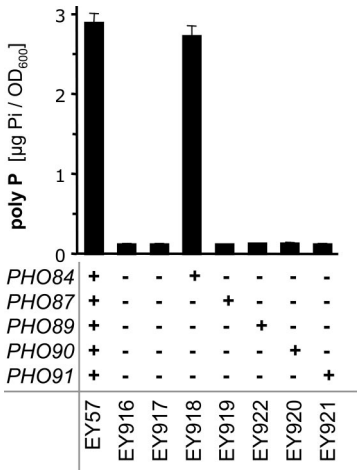

Figure 2. Poly P content in yeast strains containing none or only one of the five phosphate transporters. The strains were precultured in YPGal medium, and poly P experiments were performed in YPD medium. The figure shows average values and standard deviations of three independent samples that were harvested 2 h after the transfer to YPD medium.

It is also known that the deletion of *PHO85* results in the constitutive up-regulation of the *PHO* pathway and that *PHO84* is one of the most strongly up-regulated genes (Ogawa *et al.,* 2000). Therefore, we tested if the poly P hyperaccumulation phenotype of the *pho85*Δ strains correlated with up-regulation of *PHO84*. Poly P content in the double deletion strain of *PHO84* and *PHO85* was strongly reduced and lower than in the strain lacking only *PHO84* (Figure 3). We therefore conclude that up-regulation of Pho84 is essential for poly P hyperaccumulation in the *pho85*Δ strain. In addition to *PHO84,* we also tested the influence of the other four phosphate transporters in a *pho85*Δ strain background. Because these transporters, except Pho87, contribute only minimally to phosphate uptake and showed a weaker poly P phenotype, it was expected that these four double deletion strains would have the same poly P phenotype as the strain with an inactivated *PHO85* gene. Unexpectedly, the deletion of the different phosphate transporters in the strain lacking *PHO85* had a much stronger effect on poly P levels than that observed in the single deletions of the five transporters (Figure 3). The poly P levels in the double deletions strains of *PHO85* and *PHO89* were

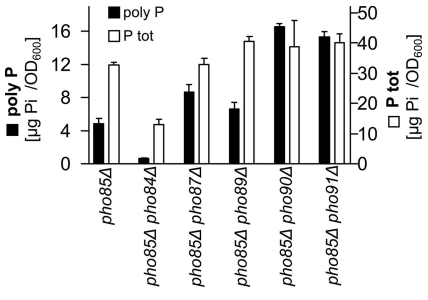

Figure 3. Low-affinity phosphate transporters limit poly P accumulation in a *pho85*Δ strain background. Poly P content (■) and total phosphate levels (P tot, □) in double deletion strains of *PHO85* and *PHO84*, *PHO87*, *PHO89*, *PHO90,* or *PHO91* were determined in quadruplet samples (harvested at the 4-h time point from cultures grown in YPD), and the standard deviations are indicated.

little affected, but the deletion of *PHO87, PHO90,* or *PHO91* in the *pho85*Δ strain background caused an increase of poly P levels (Figure 3). The *pho85*Δ *pho87*Δ strain usually showed slightly increased poly P levels by a factor of 1.5–2, while the double deletion of *PHO85* with either *PHO90* or *PHO91* caused 3 - 4 times higher poly P content as compared with the *pho85*Δ strain (Figure 3). Contrary to the poly P levels, total phosphate content in these strains changed less. Consequently, the *pho85*Δ *pho87*Δ, *pho85*Δ *pho90*Δ, and the *pho85*Δ *pho91*Δ strains stored more of their total phosphate as poly P (Figure 3). From these results we conclude that the low-affinity phosphate transporters suppress poly P levels in cells with a constitutively up-regulated *PHO* pathway, i.e., in *PHO85* mutant cells.

Increased poly P Levels in Double Deletion Strains Are Not Due to the Up-Regulation of the PHO *Pathway or to Posttranslational Effects on Pho84*

The single deletion strains of *PHO90* and *PHO91* were least affected in their poly P accumulation, and in the *pho85*Δ background all three low-affinity phosphate transporters (Pho87, Pho90, and Pho91) limited poly P levels (Figure 1). Therefore, we further characterized their effect on the metabolism of phosphate and poly P. The inhibitory effect of Pho87, Pho90, and Pho91 on poly P levels in the *pho85*Δ strain could have been caused by a repression of the *PHO* pathway and Pho84 levels or localization. As a measure for the activation of the *PHO* pathway, we determined rAPase activity in the single and double deletion strains that were used for the poly P measurements. In comparison to wild type, and as shown previously (Huang and O'Shea, 2005), rAPase activity was strongly increased in the *pho85*Δ strain, but only insignificantly higher in the *pho87*Δ and *pho91*Δ strains (Figure 4A). The double deletion strains of *PHO90* or *PHO91* with *PHO85* secreted very similar rAPase levels as the *pho85*Δ strain and only the *pho85*Δ *pho87*Δ double deletion strain exhibited minimally reduced rAPase activity (Figure 4A). Neither did the double deletion of *PHO85* and *PHO87*, *PHO90* or *PHO91* significantly alter transcription from the *PHO84* promoter as shown by β-galactosidase activities in a LacZ-reporter assay (determined with the actin [*ACT1*] promoter as a reference; Figure 4B). However, the *pho87*Δ and *pho90*Δ and to a lesser extent also the *pho91*Δ single deletion strains showed strongly up-regulated transcription from the *PHO84* promoter, even though these strains did not accumulate more poly P. This suggests that the low-affinity phosphate transporters influence *PHO84* transcription by still unknown mechanism. Because Pho84 is regulated posttranslationally by internalization and degradation in the vacuole, it was tested if Pho91 represses the effect of *PHO84* overexpression or affects Pho84 localization at the plasma membrane. The overexpression of *PHO84* in a *pho91*Δ strain did not cause a stronger poly P increase (compared with the strain with the empty plasmid) than in the wild type (Figure 4C). Overexpression of *PHO84* in the other two single deletion strains or in the *pho84*Δ strain restored wild-type poly P levels (Figure 4C). Finally, Pho84 localization at the plasma membrane was not abolished by the deletion of *PHO87, PHO90,* or *PHO91.* Pho84-GFP could be detected at the plasma membrane, inside the vacuole and presumably within the endoplasmic reticulum in cells grown in low-phosphate and in normal YPD medium, albeit the latter resulted in a much weaker signal in all compartments. However, Pho84 was slightly less frequently and less strongly internalized in the vacuole in the strains lacking *PHO90* or *PHO91* compared with the wild-type and the *pho87*Δ strain (Figure 5). On the other hand, the deletion of *PHO87* had no

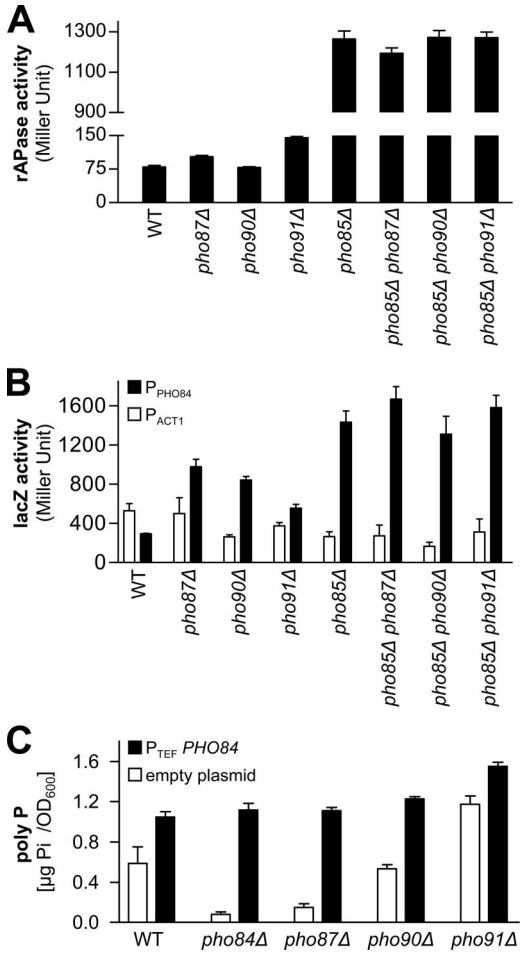

Figure 4. Deletion of *PHO87*, *PHO90,* or *PHO91* in the *pho85*Δ background does not significantly affect the *PHO* pathway. (A) rAPase activity in single or double deletion strains was determined as a measure for the activity of the *PHO* pathway. All strains were precultured in YPD medium (3d), inoculated into fresh YPD medium, and harvested after 4 h. Four measurements were taken, and average values with their SD are represented. (B) *PHO84* expression was assayed with LacZ-reporter constructs. β-Galactosidase activity is shown for the *ACT1* promoter (□) and the *PHO84* promoter (■). All strains were precultured for 2 d in SC-URA medium, transferred to low-phosphate SC-URA medium (100 μM Pi, 1d), and then inoculated into fresh YPD medium. Three independent transformants were assayed (at the 4-h time point), and the average values and standard deviations are shown. (C) Overexpression of *PHO84* from the plasmid pRS416-TEF-HA-*PHO84* in the wild-type and the deletion strains of *PHO84*, *PHO87, PHO90,* and *PHO91*. The poly P content in strains that were transformed with the empty plasmid pRS416-TEF-HA (□) or with the plasmid containing *PHO84* (pRS416-TEF-HA-*PHO84*) (■). All strains were precultured in SC-URA medium and were measured after growing for 4 h in YPD medium. The bars represent average values of four extractions, and quantifications and the SD is represented.

apparent effect on the localization of Pho84-GFP under the conditions tested. Based on the rAPase measurements, the reporter gene assays and Pho84 localization, it was concluded that the low-affinity phosphate transporters Pho87, Pho90, and Pho91 only lightly affect the *PHO* pathway, *PHO84* expression, and *PHO84* function. This small effect on the *PHO* pathway seemed unlikely to account for the large poly P increase that was observed in the *pho85*Δ strain upon the deletion of *PHO90* or *PHO91*.

The Low-Affinity Transporter Pho91 Is Localized in the Vacuolar Membrane

Because the low-affinity phosphate transporters Pho87, Pho90, and Pho91 affected poly P metabolism and phosphate allocation within the cell without clearly affecting the *PHO* pathway, we hypothesized that either of these transporters could be involved in intracellular phosphate transport. To test if any of the low-affinity phosphate transporters functions as an intracellular phosphate transporter and thereby influences poly P metabolism, we determined the localization of these three proteins by overexpressing N-terminal GFP fusions under the control of the strong ADH, the TEF and the GPD promoters, respectively. This approach was chosen because we could only observe extremely faint staining with the C-terminal GFP fusions created by Huh *et al.* (2003). In agreement with the assumed function as a part of a low-affinity phosphate uptake and/or sensing system, Pho87 and Pho90 localized to the plasma membrane independent of the promoter that was used (Figure 6). In contrast, Pho91 showed a distinct localization in intracellular membranes, which colocalized with FM4-64 staining (Figure 6). This colocalization of the FM4-64 signal with the staining from the GFP-Pho91 fusion protein placed the Pho91 transporter in the vacuolar membrane. GFP-Pho91 was always detected in the vacuole, irrespective of the promoter used and even when expressed from the weak CYC1 promoter (Figure 6). Furthermore, the vacuolar localization of Pho91 was independent of Pho86 (Figure 6), Pho87 or Pho90 (not shown). We therefore concluded that Pho91 is a vacuolar phosphate transporter and that the vacuolar localization was not an artifact of overexpression. In addition to the signal from the vacuolar periphery, we also observed bright fluorescence from clusters in or at the vacuolar membrane, mostly between adjacent vacuoles (Figure 6). These clusters became more abundant as the yeast cultures approached stationary phase and were less frequently found in cells grown in SC medium compared with YPD. Brightly stained clusters were also more abundant in a *pho85*Δ background (Figure 6). However, despite the numerous clusters at interfaces between adjacent vacuoles or vesicles in the *pho85*Δ strain, the GFP-Pho91 signal was still detected from the entire vacuolar periphery. These clusters could also have been an artifact from overexpression because they were less frequent if GFP-Pho91 was expressed from the weak *CYC1* promoter. To test if the N-terminally GFP-tagged Pho91 protein was still functional, poly P was quantified in the wild-type and *pho85*Δ strains with and without the GFP tag (Figure 7). The poly P content in the *PHO85* deletion strain containing the GFP-tagged Pho91 transporter increased only slightly, which indicated a functional fusion protein (Figure 7). To the contrary, the promoter exchange alone and introduction of the GFP-Pho91 fusion protein in the wild type rather reduced poly P levels (Figure 7), supposedly due to the presumptive overexpression of *PHO91*. This effect was much more apparent in the wild-type background (Figure 7). From these localization results it was thus concluded that active Pho91 is localized in the vacuolar membrane.

DISCUSSION

Poly P levels in yeast depend on many cellular pathways and functions such as energy metabolism, vesicle trafficking, the vtc complex, and vacuolar functions (Ogawa *et al.*, 2000; Freimoser *et al.*, 2006). Because of the obvious importance of phosphate uptake for the synthesis of poly P, we chose the phosphate transporters and the *PHO* pathway as a starting

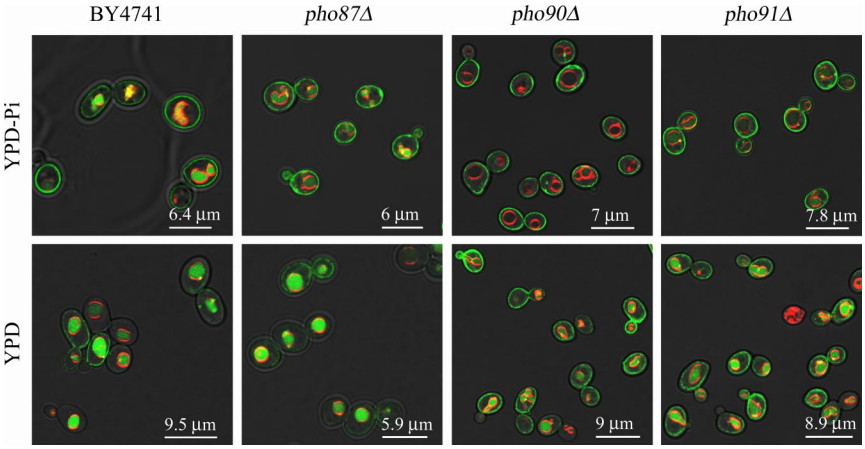

Figure 5. Pho84 localization at the plasma membrane is not abolished by the deletion of *PHO87*, *PHO90*, or *PHO91*. Localization of C-terminal GFP fusions of Pho84 in the BY4741, *pho87*Δ, *pho90*Δ, and *pho91*Δ strain backgrounds by confocal laser scanning microscopy. The four strains were either grown in YPD-Pi or in normal YPD medium, and vacuolar membranes were specifically stained with the dye FM4-64. Fluorescence intensity of the different mutant strains and of the cells from YPD and YPD-Pi medium cannot be compared. The laser power level was adjusted in order to visualize the Pho84-GFP fusion protein in strains with weak signal (in YPD medium, particularly in the BY4741 and the *pho87*Δ strain).

point to disentangle this complex regulation of the poly P metabolism in yeast.

To test if the five yeast phosphate transporters differentially affect poly P metabolism, we compared poly P levels in the single deletion strains of the five transporters and in strains that contain only one of all five transporters. In all media tested, the inactivation of *PHO84* caused the lowest poly P total, roughly half that of the wild-type strain, and only Pho84 (and none of the other transporters) conferred a normal poly P state if present as the only phosphate transporter. Thus, Pho84 is the most important phosphate transporter for poly P metabolism; irrespective of the growth medium, growth stage, or pH of the medium (not shown).

Next, we analyzed the poly P state after deletion of either one of the five phosphate transporters in the background of a *pho85*Δ strain. It had previously been shown that constitutive up-regulation of the *PHO* pathway, by the deletion of *PHO85*, causes strongly increased poly P levels (McDonald *et al.*, 2001). This increase was completely abolished by the additional deletion of *PHO84*, which suggested that up-regulation of *PHO84* was required for poly P hyperaccumulation in the *pho85*Δ strain. However, deletion of many other

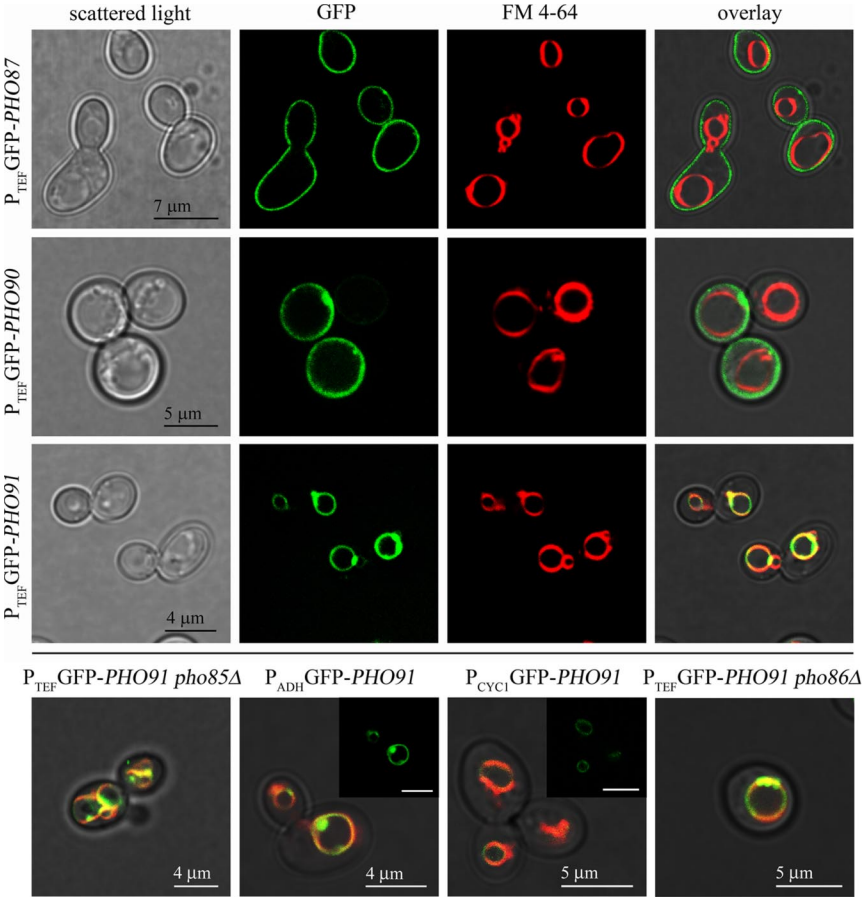

Figure 6. The low-affinity transporter Pho91 is localized in the vacuolar membrane. Localization of N-terminal GFP fusions of Pho87, Pho90, and Pho91 by confocal laser scanning microscopy. The N-terminal GFP tag, a selection marker (natMX4) and the *TEF* (strong), *ADH* (intermediate), or *CYC1* (weak) promoter were integrated into the genome by homologous recombination. Cells were grown in YPD, and as a specific marker for the vacuolar membrane, the dye FM4-64 was used. The bottom row shows only the overlay pictures of the cells expressing GFP-*PHO91* from the *TEF* promoter in the *pho85*Δ or the *pho86*Δ background or in the wild type under the control of the *ADH* and the *CYC1* promoters. For the latter two images the GFP channel is shown as an inlay (with the same scale bar).

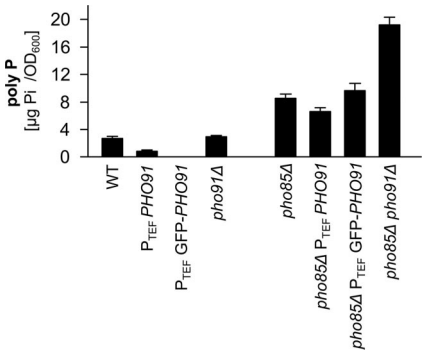

Figure 7. N-terminally GFP-tagged Pho91 is functional. The poly P content of wild-type and *pho85*Δ strains that express either untagged or the GFP-tagged Pho91 under the control of the TEF promoter was compared with the poly P levels of wild type, *pho91*Δ, *pho85*Δ, and *pho85*Δ *pho91*Δ. Yeast cultures were grown in YPD medium and quadruplet samples were harvested after 4 h. The bars represent average values of four poly P extractions, and quantifications and the SD is represented.

genes that are also involved in poly P metabolism, for example, the *VTC* genes, also abolishes or diminishes poly P hyperaccumulation in the *pho85*Δ strain (data not shown). Surprisingly, the *pho84*Δ *pho85*Δ double deletion strain contained less poly P than the *pho84*Δ single deletion strain. This suggests that some of the (numerous) effects of a *PHO85* deletion reduce poly P levels but that this phenotype is masked by the drastically increased phosphate uptake due to the up-regulation of *PHO84*. If Pho84 is indeed the determining transporter for poly P metabolism, the removal of any one of the other transporters (*PHO87*, *PHO89*, *PHO90,* or *PHO91*) in the *pho85*Δ strain should not suppress the poly P hyperaccumulation caused by the deletion of *PHO85*. Unexpectedly, combination of the *PHO87*, *PHO90,* or *PHO91* inactivation with the *PHO85* deletion strongly increased poly P hyperaccumulation compared with the *pho85*Δ strain. Although deletion of *PHO90* and *PHO91* did not have a significant effect on poly P content in the single deletion strains, it caused strong and highly significant poly P hyperaccumulation in the *pho85*Δ background. Interestingly, the double deletion strains also showed an altered phosphate allocation: The total phosphate content increased much less than the poly P content and consequently the proportion of phosphate fixed as poly P was ~2–4 times higher than in the single deletion strains. Next, we attempted to further characterize the involvement of all three low-affinity transporters in phosphate and poly P metabolism.

The double deletion strains (*pho85*Δ *pho87*Δ, *pho85*Δ *pho90*Δ, and *pho85*Δ *pho91*Δ) had neither a strongly altered rAPase activity nor did they up-regulate *PHO84*. In addition, Pho84 localization at the plasma membrane, and internalization in the vacuole was not affected by *PHO87* and only slightly dependent on *PHO90* or *PHO91*. The *pho90*Δ and *pho91*Δ strains showed slightly less internalized Pho84 than in the wild type or in the strain lacking *PHO87*. This suggests that the increased poly P hyperaccumulation was not caused by a general up-regulation of the *PHO* pathway. In contrast, Pho90 and Pho91 affected the relative poly P content and must therefore be involved in intracellular phosphate allocation. However, a triple deletion of *PHO85*, *PHO90,* and *PHO91* did not result in a further increased poly P content compared with the *pho85*Δ *pho90*Δ and the *pho85*Δ *pho91*Δ double deletion strains (not shown). Consequently, Pho90 and Pho91 do not perform redundant functions and must exert their effect on poly P metabolism by different mechanisms. We therefore conclude that Pho90 and Pho91 act independently in the regulation of phosphate metabolism or in intracellular phosphate transport.

Within the context of the global analysis of all yeast proteins Huh *et al.* (2003) localized Pho90 and Pho91 to the ER, and Pho87 could not be localized definitively. Therefore, there is little experimental evidence to support the widely held view that Pho87, Pho90, and Pho91 function in phosphate uptake from the environment across the plasma membrane (Wykoff and O'Shea, 2001; Auesukaree *et al.*, 2003; Giots *et al.*, 2003; Pinson *et al.*, 2004). Because our poly P data could be explained by an intracellular localization and functioning of either Pho87, Pho90, or Pho91, localization of these low-affinity phosphate transporters was specifically addressed.

Although Pho87 and Pho90 were indeed clearly localized to the plasma membrane, the GFP-Pho91 fusion protein revealed a clear and unmistakable localization in the vacuolar membrane. It is therefore suggested that Pho91 serves as a vacuolar phosphate transporter that exports P_i from the vacuolar lumen to the cytosol. According to this model, a deletion of *PHO91* blocks phosphate export from the vacuole, which could in turn impair degradation of the vacuolar poly P pool. A *pho85*Δ *pho91*Δ strain would thus not take up more phosphate compared with the *pho85*Δ single deletion strain, but more phosphate would be "trapped" in the form of poly P because of the reduced poly P degradation and phosphate export. This hypothesis also implies that the vacuolar poly P pool does not exert a strong negative feedback regulation on poly P synthesis in strains lacking *PHO91*. In contrast to Pho91, the observation that Pho90 limits poly P accumulation cannot be explained by its action as a phosphate transporter per se, because it was clearly localized in the plasma membrane, where it would be involved in phosphate uptake. At present, it is hypothesized that Pho90 must have a regulatory or sensory function that is involved in controlling phosphate allocation. Indeed, Pho87 or Pho90 and Pho91 have been suggested to serve as components of an external phosphate sensor (Giots *et al.*, 2003; Pinson *et al.*, 2004). However, more detailed studies will be necessary to identify the exact function of Pho87 and Pho90 in poly P metabolism.

In summary, we have shown that among the five described yeast phosphate transporters, Pho84 is the most important protein for poly P metabolism. In the absence of Pho84, poly P levels were reduced by at least 50%. Surprisingly, the low-affinity transporters Pho87, Pho90, and Pho91 negatively regulate poly P levels, which was most apparent in a *pho85*Δ background with a constitutively up-regulated *PHO* pathway. We could not detect a direct regulatory influence of Pho91 on the *PHO* pathway or on Pho84. Instead, it was found that Pho91 localizes to the vacuolar membrane. We therefore suggest that Pho91 is an intracellular phosphate transporter that affects poly P levels by regulating intracellular phosphate allocation and organellar phosphate levels.

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