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# A novel phosphatidylinositol(3,4,5)$P_3$ pathway in fission yeast

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The mammalian tumor suppressor, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), inhibits cell growth and survival by dephosphorylating phosphatidylinositol-(3,4,5)-trisphosphate (PI[3,4,5]$P_3$). We have found a homologue of PTEN in the fission yeast, *Schizosaccharomyces pombe* (*ptn1*). This was an unexpected finding because yeast (*S. pombe* and *Saccharomyces cerevisiae*) lack the class I phosphoinositide 3-kinases that generate PI(3,4,5)$P_3$ in higher eukaryotes. Indeed, PI(3,4,5)$P_3$ has not been detected in yeast. Surprisingly, upon deletion of *ptn1* in *S. pombe*, PI(3,4,5)$P_3$ became detectable at levels comparable to those in mammalian cells, indicating that a pathway exists for synthesis of this lipid and that the *S. pombe ptn1*, like mammalian PTEN, suppresses PI(3,4,5)$P_3$ levels. By examining various mutants, we show that synthesis of PI(3,4,5)$P_3$ in *S. pombe* requires the class III phosphoinositide 3-kinase, vps34p, and the phosphatidylinositol-4-phosphate 5-kinase, its3p, but does not require the phosphatidylinositol-3-phosphate 5-kinase, fab1p. These studies suggest that a pathway for PI(3,4,5)$P_3$ synthesis downstream of a class III phosphoinositide 3-kinase evolved before the appearance of class I phosphoinositide 3-kinases.

## Introduction

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was originally cloned as a tumor suppressor for gliomas (Li et al., 1997; Steck et al., 1997). We now know that PTEN is deleted or inactivated in many tumor types, including endometrial, breast, melanoma, and prostate (Simpson and Parsons, 2001). The PTEN protein is a phosphoinositide phosphatase specific for the D-3 position of the inositol ring (Maehama and Dixon, 1998). Although PTEN can dephosphorylate PI(3)P, PI(3,4)$P_2$ or PI-3,4,5-trisphosphate (PI[3,4,5]$P_3$), it is likely that PI(3,4,5)$P_3$ is the main substrate in vivo. PI(3,4,5)$P_3$ synthesis occurs via phosphorylation of PI(4,5)$P_2$, a reaction that is catalyzed by class I PI 3-kinases (Hinchliffe and Irvine, 1997). PI(3,4,5)$P_3$ activates the Akt kinases, the Tec kinases, and several small G proteins, thereby, stimulating cell motility, proliferation, and survival (Cantley, 2002).

Although classes I, II, and III PI 3-kinases are widely expressed in metazoa, only a single PI 3-kinase gene, *vps34*, has been identified in yeast (Takegawa et al., 1995). Unlike the class I enzymes, vps34p synthesizes PI(3)P but not PI(3,4)$P_2$ or PI(3,4,5)$P_3$. PI(3)P is involved in the control of vesicle trafficking to the vacuole (Odorizzi et al., 2000). The failure to detect PI(3,4,5)$P_3$ (or PI(3,4)$P_2$) in yeast is consistent with the lack of a class I PI 3-kinase and has led to the assumption that no biosynthetic pathway for PI(3,4,5)$P_3$ exists in fission or budding yeast. Our observation that the *Schizosaccharomyces pombe* ptn1p has high homology to the mammalian PI(3,4,5)$P_3$ phosphatase, PTEN, led us to question this assumption. We find that ptn1p, like its mammalian orthologue, is a PI(3,4,5)$P_3$ phosphatase. *Ptn1* disrupted (*ptn1*Δ) cells have levels of PI(3,4,5)$P_3$ comparable to mammalian

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Abbreviations used in this paper: EMM, Edinburgh minimal medium; PI, phosphatidylinositol; PI(3,4,5)$P_3$, PI-3,4,5-trisphosphate; PIP 5-kinase, PI-4-phosphate 5-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; *ptn1*Δ, *ptn1* disrupted.

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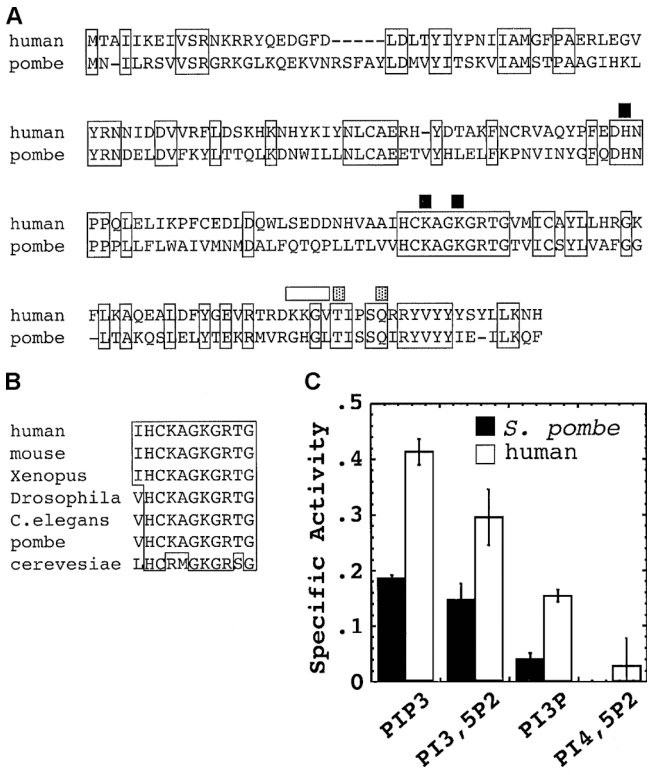


Figure 1. **Comparison of *S. pombe* PTEN proteins.** (A) Alignment of human and *S. pombe* proteins (DNA Star program, J. Hein method). Open box, 4–amino acid insertion; shaded box, residues forming H-bonds with PI(3,4,5)$P_3$; black box, positive charges at active site. (B) Signature sequences for seven species. *S. pombe* protein is similar to PTEN in higher organisms. (C) Human and *S. pombe* PTEN phosphatases have similar specificities for PI substrates. Specific activities are reported as moles of phosphate released per mole of GST-PTEN per minute. Error bars are ± SEM.

cells, display irregularly shaped vacuoles and are osmotically fragile. PI(3,4,5)$P_3$ synthesis in *S. pombe* required vps34p and its3p, but not fab1p. These results suggest a novel biosynthetic pathway for PI(3,4,5)$P_3$ that evolved before the appearance of class I PI 3-kinases.

## Results

### Identification of an *S. pombe* PTEN

Using a BLAST search, we identified an *S. pombe* gene (GenBank/EMBL/DDBL accession no. CAA22831) with significant homology to the mammalian PTEN. The putative phosphatase domain is 38% identical to the human PTEN phosphatase domain (Fig. 1 A). However, PTEN is closely related to dual specificity protein phosphatases that act on phosphoserine and phosphotyrosine, and the *S. pombe* gene might encode a dual-specificity phosphatase. Based on the crystal structure of human PTEN (Lee et al., 1999), the residues required for PI(3,4,5)$P_3$ hydrolysis have been identified. Human PTEN has a 4–amino acid (amino acids 163–166) insert (relative to dual-specificity phosphatases) that increases the size of the active site. The *S. pombe* protein also has a 4–amino acid insert (Fig. 1 A, open box above sequence). Although the *S. pombe* insert is not similar to the human sequence, it places thr-167 and gln-171 (Fig. 1 A, hatched boxes) in frame with the corresponding human sequence. These two residues form hydrogen bonds with the PI(3,4,5)$P_3$ phosphates. The basic character of the human PTEN active site results from his-93, lys-125, and lys-128. Fig. 1 A (black boxes) shows that in the *S. pombe* sequence these residues are conserved. Finally, we examined the phosphatase signature sequence (also known as the P loop), which is a critical determinant of phosphatase specificity (Fig. 1 B). The human PTEN signature sequence is identical to mouse and *Xenopus*, and differs from *Drosophila*, *C. elegans*, and *S. pombe* proteins by a single isoleucine to valine substitution. In contrast, the *Saccharomyces cerevisiae* PTEN homologue is substantially different from human with four substitutions, consistent with reports that it does not hydrolyze phosphatidylinositol (PI) phosphates (Heymont et al., 2000; Maehama et al., 2001). Hence, the human and *S. pombe* genes are homologous, and the residues essential for PI(3,4,5)$P_3$ phosphatase activity are all conserved.

To test whether the *S. pombe* protein is a PTEN orthologue, we expressed the *S. pombe* gene product in bacteria and tested it for phosphoinositide phosphatase activity. The specificity of the *S. pombe* putative phosphatase was similar to that of human PTEN. Both enzymes hydrolyzed PI(3,4,5)$P_3$, PI(3,5)$P_2$, and PI(3)P (Fig. 1 C). There was little or no activity toward PI(4,5)$P_2$. Based on the sequence homology and the phosphoinositide phosphatase activity, we conclude that this gene product is a functional orthologue of mammalian PTEN, which we designated ptn1p.

### Ptn1p affects phosphoinositide levels in vivo

To test the role of *ptn1* in vivo, we prepared a yeast strain (*ptn1*Δ) lacking *ptn1* and then introduced a pREP1 ptn1p expression vector. These yeast strains were labeled with [$^3$H]inositol, the lipids were extracted and deacylated, and the levels of phosphoinositide were analyzed by HPLC. The *ptn1*Δ cells had six- to eightfold increased levels of PI(3,4)$P_2$ and PI(3,4,5)$P_3$ as compared with wild-type cells (Fig. 2). Restoration of ptn1p levels with a ptn1p expression vector lowered PI(3,4)$P_2$ and PI(3,4,5)$P_3$ levels close to wild-type levels. Manipulation of ptn1p levels did not affect PI(3)P levels, indicating that in vivo PI(3)P is not a significant substrate for ptn1p, as has been suggested for human PTEN (Leslie and Downes, 2002). These results confirm that ptn1p is a PI(3,4,5)$P_3$ phosphatase in vivo and provide the first report of PI(3,4,5)$P_3$ in yeast.

### Synthesis of PI(3,4)$P_2$ and PI(3,4,5)$P_3$ in *S. pombe* involves vps34p and its3p

In mammalian cells, the main pathway for PI(3,4,5)$P_3$ synthesis involves phosphorylation of PI(4,5)$P_2$ by a class I PI 3-kinase. Using BLAST searches of the *S. pombe* genome, we were not able to identify any putative class I PI 3-kinase genes, which are consistent with the experimental finding that vps34p is the only PI 3-kinase in yeast (Takegawa et al., 1995). To determine whether vps34p is critical for PI(3,4,5)$P_3$ production, we crossed a *vps34*Δ line (Takegawa et al., 1995) with *ptn1*Δ. The resulting line *vps34*Δ *ptn1*Δ had greatly reduced levels of PI(3,4)$P_2$ and PI(3,4,5)$P_3$ (Fig.

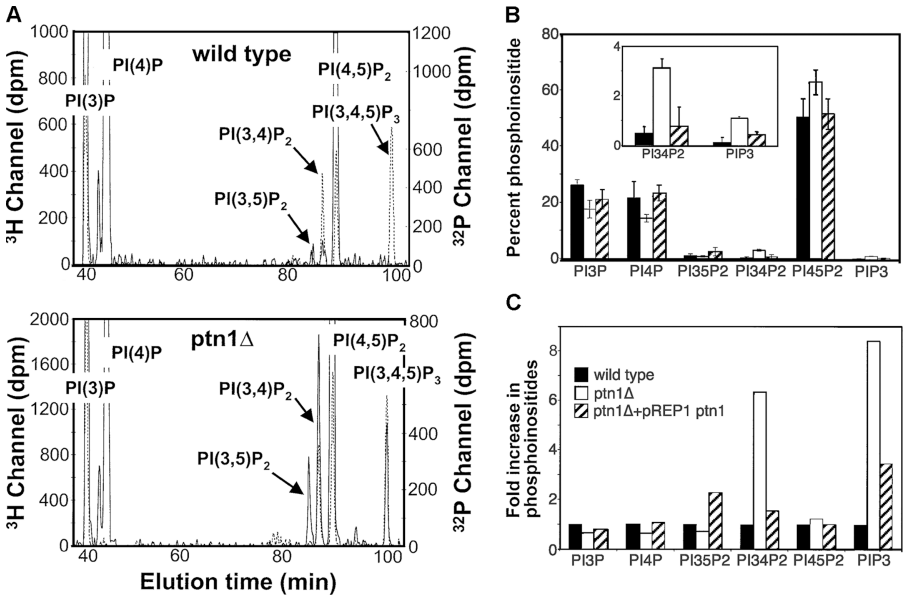


Figure 2. **Ptn1p affects PI levels in cells.** (A) HPLC profiles of [$^3$H]inositol-labeled phosphoinositides from wild-type and *ptn1*Δ cells (solid lines). $^{32}$P-labeled lipids were used as internal standards (dashed lines). (B) Quantification of phosphoinositide levels in wild-type cells, *ptn1*Δ cells, and *ptn1*Δ cells transformed with pREP1 ptn1p expression vector (*ptn1*Δ+pREP1 ptn1p). The data were normalized to give the sum of PI phosphates a value of 100%. We used this method because the incorporation of [$^3$H]inositol into PI relative to PI phosphates varied considerably from sample to sample (11.0 ± 6.7, average ± SD). The inset shows a magnified view of the values for PI(3,4)$P_2$ and PI(3,4,5)$P_3$. (C) Fold increase in phosphoinositide levels as compared with the levels in wild-type cells. The data were normalized to give each wild-type phosphoinositide a value of 1.

3 A). We also tested whether fab1p (Gary et al., 1998), a lipid kinase that converts PI(3)P to PI(3,5)$P_2$ is involved in PI(3,4,5)$P_3$ synthesis. The *fab1*Δ *ptn1*Δ line lacked PI(3,5)$P_2$ but had PI(3,4)$P_2$ and PI(3,4,5)$P_3$ levels comparable to the *ptn1*Δ line (Fig. 3 B). These results demonstrate that vps34p, but not fab1p, is essential for production of PI(3,4)$P_2$ and PI(3,4,5)$P_3$ in *S. pombe*.

In mammalian cells, there is an alternative route for PI(3,4,5)$P_3$ synthesis (Zhang et al., 1997; Tolias et al., 1998; Halstead et al., 2001). A PI-4-phosphate 5-kinase (PIP 5-kinase) mediates the conversion of PI(3)P to PI(3,4)$P_2$ as well as the subsequent conversion to PI(3,4,5)$P_3$. Its3p is the major PIP 5-kinase in *S. pombe* (Zhang et al., 2000) and, therefore, might play a role in the synthesis of PI(3,4,5)$P_3$. Because disruption of *its3* is lethal, we used the *its3-1* line, which has ~10% of wild-type PIP 5-kinase activity (Zhang et al., 2000). We crossed the *its3-1* cells with *ptn1*Δ cells and assayed phosphoinositide levels (Fig. 3 C). As expected, PI(4,5)$P_2$ levels were decreased in *its3-1* and *its3-1 ptn1*Δ cells. In addition, PI(3,4)$P_2$ and PI(3,4,5)$P_3$ levels were decreased in *its3-1 ptn1*Δ cells compared with *ptn1*Δ cells. The data are consistent with a model in which its3p, like its mammalian homologue, can convert PI(3)P to PI(3,4)$P_2$ and PI(3,4,5)$P_3$.

## Ptn1p affects vacuole morphology and osmotic fragility

The *ptn1*Δ cells grew normally and had a normal morphology by bright field microscopy. However, using EM, we found that the *ptn1*Δ cells had misshapen vacuoles (Fig. 4). To quantify this phenotype, we counted the cells with at least 50% irregularly shaped vacuoles. Fig. 4 C shows that >70% of the *ptn1*Δ cells presented this phenotype. These findings demonstrate an effect of *ptn1* disruption on vacuole morphology. We analyzed the ptn1p subcellular localization with a pREP42 GFP-ptn1p expression vector. The GFP-ptn1p fusion protein was detected in both punctate structures (0.5–1.0 μm in diameter) and septa of dividing cells (Fig. 5 A). As controls, we expressed a pREP42-GFP vector or an untagged pREP1-ptn1p vector. We did not observe punctate or septal fluorescence.

We tested GFP-ptn1p activity by osmotically stressing yeast cells (Fig. 5 B). In this assay (Paravicini et al., 1992), osmotic stress leads to cell lysis, release of alkaline phosphatase and formation of a bluish-green color. Inclusion

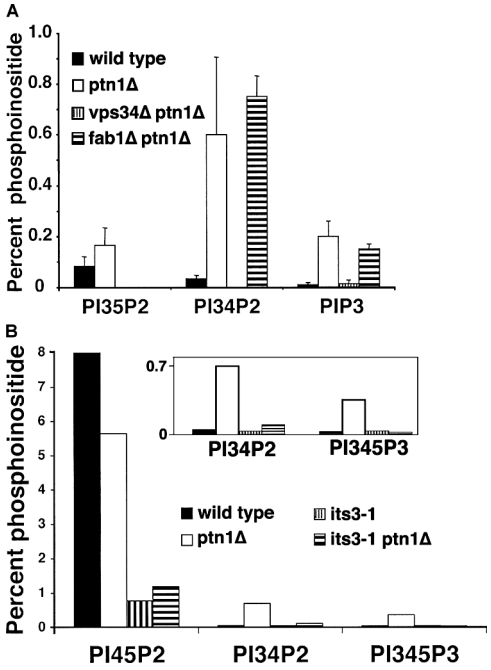


Figure 3. **PI(3,4,5)$P_3$ biosynthesis requires vps34p and its3p but not fab1p.** (A) Wild-type *S. pombe*, *ptn1*Δ, *vps34*Δ *ptn1*Δ, and *fab1*Δ *ptn1*Δ cells were labeled with [$^3$H]inositol. Phosphoinositides were extracted, deacylated, and analyzed by HPLC. Error bars are ± SEM. (B) Wild-type *S. pombe*, *ptn1*Δ, *its3-1*, and *its3-1 ptn1*Δ cells were labeled and analyzed as in A. In this series of experiments (A and B), we normalized the data in relation to PI because the *vps34* and *its3-1* mutations resulted in major and reproducible reductions in incorporation of [$^3$H]inositol into the PI phosphate pool.

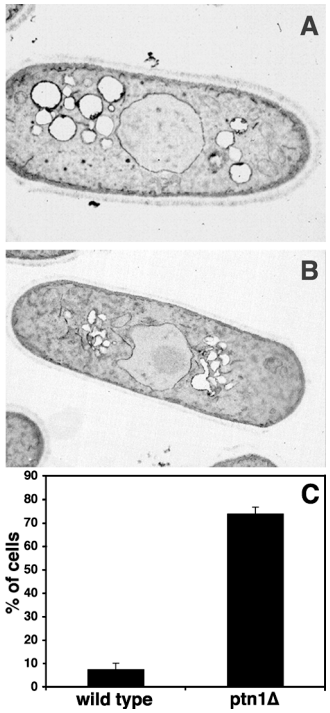


Figure 4. **Regulation of vacuole morphology by ptn1p.** Electron micrographs are shown for wild-type (A) and *ptn1*Δ cells (B). In C, the results are quantified, counting ~100 cells in each category. Cells with at least 50% misshapen vacuoles were scored as positive for this phenotype. Loss of ptn1p leads to misshapen vacuoles. Error bars are ± SEM.

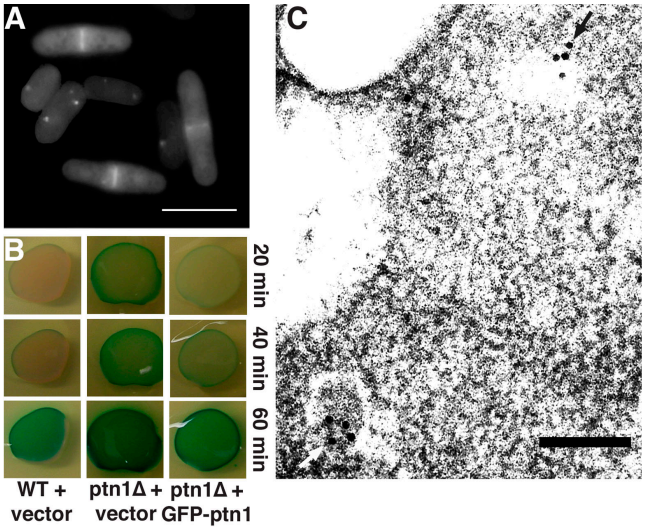


Figure 5. **Subcellular localization and activity of GFP-ptn1p.** (A) By fluorescence microscopy, GFP-ptn1p was detected in punctate structures and septa. Bar, 10 μm. (B) Wild-type *S. pombe* +pREP1 vector, *ptn1*Δ+pREP1 vector, and *ptn1*Δ+pREP1 GFP-ptn1p cells were osmotically stressed, and cell lysis was detected by the resulting bluish-green color. The *ptn1*Δ+pREP1 cells lysed more quickly than wild-type cells, and GFP-ptn1p expression largely reversed this phenotype. (C) Immuno-EM shows that the GFP-ptn1p-containing puncta are small vesicles that may be endosomes. Bar, 0.1 μm. These results were obtained with monoclonal anti-GFP, but a polyclonal anti-GFP antibody gave a similar pattern (not depicted).

of 1.2 M sorbitol inhibited color formation (unpublished data), indicating that cell lysis is, indeed, due to osmotic stress. In this assay, the *ptn1*Δ cells lysed more quickly than wild-type cells. Furthermore, expression of GFP-ptn1p largely reversed this phenotype. These results demonstrate that GFP-ptn1p is active and provide a simple, in vivo assay for ptn1p activity. Osmotic fragility is usually associated with a cell wall defect, but further experiments are required to confirm this mechanism for the *ptn1*Δ cells.

Based on the lack of colocalization of GFP-ptn1p with rhodamine phalloidin, the punctate structures were not associated with actin patches (unpublished data). To further characterize these punctate structures, we performed immuno-EM, using anti-GFP antibodies. Clusters of immunogold particles were detected in association with vesicular structures (Fig. 5 C, arrow). The gold particles were generally not associated with the larger vacuoles. Based on the size, we suspect that these structures may be endosomes (Prescianotto-Baschong and Riezman, 2002). Control cells that did not express GFP-ptn1p did not show significant numbers of immunogold particles.

We next sought a PH domain protein that binds $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$ and, thereby, mediates downstream signaling. The *S. pombe* genome includes 21 proteins with predicted PH domains (Wood et al., 2002). Based on rules developed for mammalian $PI(3,4,5)P_3$-binding PH domains (Rameh et al., 1997; Lietzke et al., 2000), we identified seven candidates and tested them for phosphoinositide binding using filters spotted with lipids. Two of these PH domain proteins showed binding to $PI(3,4,5)P_3$ in vitro, although none showed high specificity for binding to this lipid compared with $PI(4,5)P_2$. The first was a predicted protein designated SPAC 11E3.11C, which is a homologue of the ARNO/cytohesin/Grp family of Arf exchange factors (Fig. 6 A). The second was ksg1p, which is the *S. pombe* homologue of the mammalian $PI(3,4,5)P_3$ regulated kinase, PDK1 (Niederberger and Schweingruber, 1999; unpublished data). Although the in vivo binding specificity of lipid binding domains often correlates with this in vitro assay, this is not always the case (Yu et al., 2004). A more reliable assay is relocalization of the protein in vivo in response to a perturbation that alters phosphoinositide levels. In both wild-type and *ptn1*Δ cells, GFP-ksg1p showed septal and plasma membrane localization (unpublished data). A possible explanation is that the ksg1 PH domain targets the plasma membrane and septum via $PI(4,5)P_2$ rather than $PI(3,4,5)P_3$. In 83% (72/86) of *ptn1*Δ cells, the GFP-11E.11C protein localized to endosome-like structures, septa, and growing ends (Fig. 6 B), resembling the distribution of GFP-ptn1p (Fig. 5 A). In contrast, examination of >100 wild-type cells showed no clear localization of the GFP-11E3.11C protein (Fig. 6 C). These experiments establish 11E3.11C as a good candidate for a $PI(3,4)P_2$/$PI(3,4,5)P_3$–binding PH domain protein in *S. pombe*.

## Discussion

Here, we have identified ptn1p, an *S. pombe* homologue of mammalian PTEN. The ptn1p phosphatase domain is 38% homologous to the human PTEN phosphatase domain, and

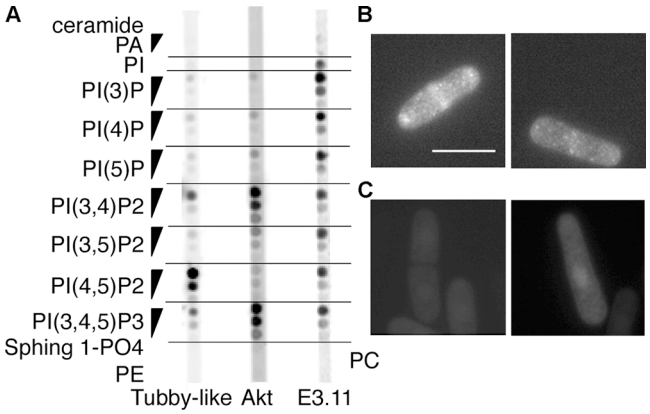


Figure 6. **Phosphoinositide-binding PH domain regulated by ptn1p.** (A) Binding of $^{35}$S-labeled PH domains to lipids immobilized on a membrane. The Tubby-like and Akt PH domains show specificity for $PI(4,5)P_2$ and $PI(3,4)P_2+PI(3,4,5)P_3$, respectively. The 11E3.11C PH domain from *S. pombe* binds many phosphoinositides, including $PI(3,4,5)P_3$. (B) Distribution of GFP-11E3.11C PH domain in *ptn1*Δ cells. The left and right panels show a dividing cell and a growing cell, respectively. Bar, 10 μm. (C) GFP-11E3.11C PH domain shows no clear localization in wild-type cells.

all of the residues essential for PIP phosphatase activity are conserved. Furthermore, recombinant ptn1p dephosphorylates $PI(3,4,5)P_3$ and cells lacking *ptn1* show markedly increased levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$. Based on these findings, we reach the surprising conclusion that *S. pombe* has a true PTEN orthologue that regulates the levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$.

The discovery of *ptn1* led us to examine the biosynthetic pathway for $PI(3,4,5)P_3$ synthesis. We discovered a novel pathway that originates with synthesis of PI(3)P by vps34p, followed by the conversion of PI(3)P to $PI(3,4,5)P_3$. Its3p, the *S. pombe* orthologue of mammalian type I PIP 5-kinases, converts PI(3)P into $PI(3,4)P_2$, as has been shown to occur for mammalian type I PIP 5-kinases (Zhang et al., 1997; Tolias et al., 1998). The enzyme that catalyzes the last step in the synthesis of $PI(3,4,5)P_3$ has not been identified, but by analogy with the mammalian pathway, may also be its3p. The observation that wild-type cells have undetectable or very low levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ indicates that, as for mammalian cells, these lipids are tightly regulated in fission yeast. This regulation may occur at the level of synthesis and/or degradation of these lipids. Here we show that the ptn1p has an important role in maintaining the low levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in *S. pombe.* Understanding the spatial and temporal regulation of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ synthesis are important questions for future studies.

Ptn1p, like PI(3)P (Gillooly et al., 2000) and vps34p (Stack et al., 1995), was found associated with vesicular structures, and *ptn1*Δ cells show irregularly shaped vacuoles and are more readily lysed by osmotic stress. However, we also observed ptn1p associated with the septa of dividing cells. Hence, as in mammalian cells, $PI(3,4,5)P_3$ (and/or $PI(3,4)P_2$) in *S. pombe* likely has multiple functions, regulating different processes in different regions of the cell.

The mechanism by which this lipid affects cell function in fission yeast remains to be determined. One can imagine that, as for mammalian cells, $PI(3,4,5)P_3$ (and/or $PI(3,4)P_2$) may function to recruit target proteins to specific subcellular locations via binding to protein modules. The *S. pombe* genome includes 21 putative PH domains (Wood et al., 2002), which in mammalian cells bind to PI phosphates and mediate many of the downstream effects. Our investigation of the phosphoinositide binding specificity of *S. pombe* PH domains revealed that the GFP-11E3.11C PH domain has distinct subcellular distributions in wild-type and *ptn1*Δ cells, suggesting that it is regulated by $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$. However, by the filter binding assay the 11E3.11C PH domain is not specific for $PI(3,4)P_2$ or $PI(3,4,5)P_3$. There are several possible explanations. First, there may be experimental complications, relating to incomplete folding of in vitro translated PH domains, thereby, compromising PH domain specificity. In addition, binding of PH domains to filters is an excellent method for surveying phosphoinositide specificity, but binding of PH domains to undiluted phosphoinositides on a filter is sometimes less selective than in biological membranes (Snyder et al., 2001). Second, specific binding of *S. pombe* PH domains to membranes might require interactions with both lipid and protein targets. Indeed, some *S. cerevisiae* PH domains require multiple interactions for membrane binding (Yu et al., 2004). Third, localization of the 11E3.11C PH domain in *ptn1*Δ cells may be due to a higher affinity for $PI(3,4,5)P_3$ than $PI(4,5)P_2$, as has been observed for the ARNO PH domain (Venkateswarlu et al., 1998; Cullen and Chardin, 2000). The 11E3.11C predicted protein is a homologue of the ARNO/cytohesin/Grp family and like these mammalian proteins, has an Arf GDP/GTP exchange domain and PH domain. Hence, $PI(3,4,5)P_3$ in lower eukaryotes may act through a PH domain (domains) that binds multiple phosphoinositides, and $PI(3,4,5)P_3$-specific PH domains may have evolved in more complex species.

In summary, the results presented here indicate that a pathway for the synthesis of $PI(3,4,5)P_3$ from PI(3)P existed in yeast before the evolution of class I PI 3-kinases in higher eukaryotes, indicating a more ancient function for this important signaling molecule.

## Materials and methods

### Generation of constructs, yeast strains, and recombinant proteins

The *ptn1* ORF was amplified by PCR from *S. pombe* genomic DNA and subcloned into the pREP1 expression vector containing a thiamine repressible *nmt-1* promoter. For localization studies, this PCR product was ligated to the 3′ end of a GFP cDNA in the pREP42 expression vector, which contains an attenuated version of *nmt-1* promoter. To isolate recombinant proteins, the GST fusion construct of *ptn1* was induced for 3 h in BL-21 cells, and the resulting fusion proteins were purified from bacterial lysates using glutathione Sepharose (Amersham Biosciences). All plasmids were verified by automated DNA sequencing. The *ptn1*Δ fission yeast strain was prepared by standard one step homologous recombination mediated gene replacement method. Stable integrants were selected in medium lacking uracil, and disruption of the gene was checked by PCR analysis. The *vps34*Δ (Takegawa et al., 1995), *fab1*Δ (Morishita et al., 2002), and *its3-1* (Zhang et al., 2000) fission yeast strains were gifts from K. Takegawa (Kagawa University, Kagawa, Japan), C. Shimoda (Osaka City University, Osaka, Japan), and T. Kuno (Kobe University School of Medicine, Kobe, Japan). These strains were crossed with the *ptn1*Δ strain to generate appropriate double mutants.

### Phosphatase assay

GST-PTEN and GST-ptn1 (1 μg/assay) were incubated with 25 nmoles of appropriate dioctanoyl PI substrates in 500 μl of assay buffer, containing

50 mM Tris-HCl, pH 7.5, and 2 mM DTT for 30 min at 37°C. The reaction was stopped by addition of malachite green solution (BIOMOL Research Laboratories, Inc.), and the enzyme activity was measured by the change in absorption at 650 nm using appropriate controls.

### In vivo analysis of phosphoinositides

Log phase cultures of yeast strains were grown in Edinburgh minimal medium (EMM) synthetic media plus appropriate supplements. Cells were washed twice in inositol-free EMM medium and subcultured ($10^6$ cells/ml) for 20 h in 5 ml of the same medium containing 10 μCi of myo[2-$^3$H]inositol. Labeled cells were harvested and lysed by vigorous vortexing with 0.5 ml 1 N HCl, 1 ml methanol-chloroform (1:1 vol/vol), and 1.5 g of acid-washed glass beads (Sigma-Aldrich). Bovine brain phosphoinositides (40 μg/sample; Sigma-Aldrich) were added as carrier lipid, and phase separation was induced by addition of 0.4 ml chloroform. The extracted lipids were deacylated and analyzed by anion exchange high pressure liquid chromatography using a Partisphere SAX column (Agilent Technologies), using an online detector (Serunian et al., 1991).

### Microscopy

For localization of GFP-ptn1, cells were grown to early log phase in EMM+adenine+leucine+thiamine, washed twice in EMM+adenine+leucine medium, and induced in the same medium for 20 h. For colocalization with actin, cells were fixed in 3.7% formaldehyde, stained with rhodamine phalloidin (Molecular Probes), and visualized with a Zeiss Axioskop and Apochromat 100X objective ($n = 1.4$). Micrographs were recorded with an AxioCam digital camera and OpenLab software (Improvision). For ultrastructural analysis, early log phase cells were fixed in 2% $KMnO_4$ for 30 min at RT, washed and dehydrated in sequential grades of ethanol, embedded in Epon resin at 65°C overnight, and stained with lead citrate and uranyl acetate (Armstrong et al., 1993). Observation was based on examination of at least 100 cells. Digital images were prepared using Adobe Photoshop 7.0.

For immuno-EM, cells grown to log phase were fixed with 4% PFA plus 0.4% glutaraldehyde for 30 min at RT with shaking. The cells were then dehydrated and embedded in LR white resin. Sections were blocked in 2% BSA plus 0.2% normal goat serum. Sections were stained with anti-GFP mAb (1:100; Covance) or a polyclonal rabbit antibody (1:50; Abcam). After washing, the sections were incubated with 10 nm of anti-antibody gold particles (Jackson ImmunoResearch Laboratories). The samples were washed and fixed after with 2% glutaraldehyde. The sections were stained with 1% uranyl acetate plus 1% lead citrate. Finally, the sections were briefly exposed to osmium vapor to provide additional contrast.

### Assay for osmotic integrity of yeast cells

Yeast cells were overlaid with 0.05 M glycine HCl, pH 9.5, 1% agar, and 10 mM 5-bromo-4-chloro-3-indolyl phosphate (Paravicini et al., 1992). The cells that lysed released alkaline phosphatase and turned bluish green.

### Phosphoinositide binding by PH domains

Sequences containing the PH domains for ksg1 (residues 434-592), OBP1 (residues 254-350), OBP2 (residues 121-260), pob1 (residues 690-815), SPAC 11E311C (residues 500-942), SPAC 26A3.10 (residues 501-651), and SPBC 17G9.08C (residues 500-630) were in vitro transcribed and translated with [$^{35}$S]methionine (Promega TNT coupled transcription/translation system). The $^{35}$S-labeled proteins were incubated in 3% (wt/vol) fatty acid–free BSA (Sigma-Aldrich), 0.05% (vol/vol) Tween 20, 150 mM NaCl, 50 mM Tris, pH 7.5, with PVDF membranes spotted with phospholipids. The synthetic diC16:0 phosphoinositides were from Cell Signals, Inc. and were spotted at 180, 60, and 20 pmoles per 100 nl spot. Phosphatidic acid was spotted at 180 and 60 pmoles. Ceramide, PI, sphingosine-1-phosphate, phosphatidylcholine, and phosphatidylethanolamine were each spotted at 180 pmoles. After incubation with $^{35}$S-labeled proteins for 2 h at 4°C, the membranes were washed with 0.05% Tween 20, 150 mM NaCl, 50 mM Tris, pH 7.5. Proteins binding to lipid spots were detected by phosphorimaging.

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