Vip1 is a kinase and pyrophosphatase switch that regulates inositol diphosphate signaling

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Inositol diphosphates (PP-IPs), also known as inositol pyrophosphates, are high-energy cellular signaling cofactors involved in nutritional and regulatory responses. We report that the evolutionarily conserved gene product, Vip1, possesses autonomous kinase and pyrophosphatase domains capable of synthesis and destruction of D-1 PP-IPs. Our studies provide atomic-resolution structures of the PP-IP products and unequivocally define that the Vip1 gene product is a highly selective 1-kinase and 1-pyrophosphatase enzyme whose activities arise through distinct active sites. Kinetic analyses of kinase and pyrophosphatase parameters are consistent with Vip1 evolving to modulate levels of 1-IP₇ and 1,5-IP₈. Individual perturbations in kinase and pyrophosphatase activities in cells result in differential effects on vacuolar morphology and osmotic responses. Analogous to the dual-functional key energy metabolism regulator, phosphofructokinase 2, Vip1 is a kinase and pyrophosphatase switch whose 1-PP-IP products play an important role in a cellular adaptation.

Significance

Our studies demonstrate that Vip1 represents a rare class of bifunctional enzyme capable of synthesizing and destroying signaling molecules important for nutrient adaptation, cellular architecture, and organelle morphology. We find that Vip1 contains two tethered autonomous catalytic active sites, which modulate levels of 1-IP₇ and 1,5-IP₈ through 1-kinase and 1-pyrophosphatase domains. Each activity is critical for maintaining the highly dynamic anabolic and catabolic regulation of cellular pools of IP₇ and IP₈. That this occurs through a single gene product emphasizes that Vip1 is a key metabolic switch for inositol 1-pyrophosphate–mediated cellular signaling and adaptation.

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The authors declare no competing interest.

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Data deposition: Structural data have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes 6PCK and 6PCL).

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Results

Note. The vast majority of the work presented in this manuscript was first reported at international and Howard Hughes Medical Institute scientific meetings as well as universities beginning in the fall of 2009. While it was not published in a timely manner, which the senior author greatly regrets, at the suggestion of several colleagues in the field, the manuscript is presented here in an unorthodox retrospective manner. This is in no way meant to belittle or ignore the current state of the field, including published work of others; rather it is written in the historical context of our work in 2008 and 2009. The exceptions are as follows: 1) finalized enzymological parameters performed in February 2012, and 2) statistical analysis/quantification of the vacuole diameter, which was performed in 2019 by Kavi Mehta at Vanderbilt (Nashville, TN) on images generated in 2009.

The Vip1 Family of Kinases Phosphorylate the D-1 Phosphate of IP6. Previous studies using NMR methods aimed at identification of the product(s) of the *Saccharomyces cerevisiae* Vip1 (scVip1) kinase determined that the enzyme phosphorylated either the 1- or 3-phosphate positions (17). However, due to the 2/5 symmetry axis of the inositol ring, these studies could not differentiate between the 1- or 3- stereoisomer. We therefore initiated a structural approach in the context of a protein cocrystal to resolve chirality and isomer ambiguities. The cocrystal structures of inositol pyrophosphatase Dipp1 in complex with the IP7 species produced by scVip1 and hIP6K1 were determined at near atomic 1.2-Å resolution (Table 1 and Fig. 1). Since these IP7 species produced by scVip1 and hIP6K1 were determined at near atomic 1.2-Å resolution (Table 1 and Fig. 1). Since these IP7 species are substrates for Dipp1, low pH conditions for crystallization were used in which binding would still occur but that Dipp1 active was greatly diminished (Fig. 1A). Unambiguous electron density was found near the active site of DIPP in the scVip1 IP7 cocrystal, which clearly revealed a 1-IP7 molecule with no evidence of any other species or conformations (Fig. 1B). The high-resolution structure of the IP6K1 IP7 product/DIPP complex confirms the stereomer as unambiguously 5-IP7 (Fig. 1B).

Table 1. Crystallographic data collection and refinement statistics for Dipp1/IP7 complexes

| Diffraction data | Dipp1/1-IP7 complex | Dipp1/5-IP7 complex |
|------------------|---------------------|---------------------|
| PDB ID code      | 6PCK                | 6PCL                |
| Space group      | P2₁,2₁,2₁           | P2₁,2₁,2₁           |
| a,b,c, Å         | 46.41, 59.61, 62.56 | 45.46, 59.58, 62.36 |
| Wavelength, Å    | 1.0000              | 1.0000              |
| Resolution limit, Å* | 1.20             | 1.30                |
| Unique reflections | 51,322            | 40,069              |
| Completeness, %, (last shell) | 93.9 (68.1) | 93.7 (65.2) |
| Average I/σ₀ (last shell) | 70.7 (5.6) | 58.2 (3.5) |
| Redundancy (last shell) | 12.7 (8.4) | 12.5 (4.9) |
| R<sub>sym</sub>, % (last shell) | 6.1 (37.5) | 6.0 (36.5) |
| Crystallographic refinement | 27.4–1.2 | 27.6–1.3 |
| Reflections      | 47,532              | 38,468              |
| Rms deviation from ideality | 0.022    | 0.011               |
| Bond lengths, Å  | 1.898               | 1.374               |
| Ramachandran     | Outliers            | 0.0%                | 0.0%                |
|                  | Allowed             | 100%                | 100%                |
|                  | Favored             | 100%                | 100%                |
|                  | R value, %          | 15.7                | 14.3                |
|                  | R<sub>free</sub>, % | 17.3                | 16.1                |

*Resolution limit was defined as the highest-resolution shell where the average I/σ₀ was >1.2 and R<sub>sym</sub> < 50%.

Vip1 Is Evolutionarily Conserved Pyrophosphatase Selective for the D-1 Position. The two-domain topology of the Vip1 class of enzymes is evolutionarily conserved and has a consistent topology of an N-terminal kinase domain of ~350 amino acids followed by a HAP-like domain of ~450 amino acids (Fig. 2A). Among the HAP family members are the phytase cluster of phosphomonoesterases enzymes that hydrolyze IP₆. Initially, we tested a variety of species of the Vip1 class of HAP domains for activity toward IP₆ without success. We next tested *S. pombe* spAsp1 HAP domain (spAsp1-HAP, residues 377 to 920) for in vitro activity against a collection of additional inositol phosphates and pyrophosphates. Pyrophosphatase activity was detected that converted IP₇ to IP₆, and titration of IP₇ species against spAsp1-HAP indicated that the enzyme showed strong selectivity for the 1-IP₇ isomer produced by spAsp1 over the 5-IP₇ isomer produced by IP₆K (Fig. 2B). No phosphomonoesterase activity was observed toward other IPs, including IP₆, indicating that the pyrophosphatase activity of spAsp1 is specific for cleaving the disphosphate bond; thus, we refer to it as a 1-pyrophosphatase (E.C. 3.6.1). Maximal pyrophosphatase activity was observed at pH 5.0 and nearly 30% activity was observed at physiological pH of 6.8 (Fig. 2C). To demonstrate that the 1-pyrophosphatase activity was not due to contamination, a catalytic point mutant (spAsp1-HAP-H397A) was cloned, expressed, and purified through the same procedure as the wild-type spAsp1 pyrophosphatase domain (Fig. 2D), and evaluated for the ability to hydrolyze inositol diphosphate (IP-IP) substrates. While the wild-type spAsp1-HAP hydrolyzed 1-IP₇ to produce IP₆, no pyrophosphatase activity was detected even up to a 500-fold excess of the mutant enzyme (Fig. 2D).
The affinity of the enzyme for 1-IP7 offset by a 10-fold decrease in catalytic efficiency was a 30-fold increase in full-length spAsp1 at near-physiological pH (Table 3). This shift in turnover and catalytic efficiency are seen when comparing the catalytic efficiencies of the 1-kinase and 1-pyrophosphatase activities of Vip1 enzymes produce and destroy 1-PP-IPs at the expense of consuming ATP (a so-called futile cycle), we probed the catalytic efficiency/selectivity data demonstrate that Vip1 encodes an evolutionarily conserved class of dual-functional enzyme switches that have 1-kinase activity toward IP7 and 1,5-IP8 substrates and 1-pyrophosphatase activities toward the kinase products 1-IP7, 1,5-IP8, and 1-IP8 (schematically shown in Fig. 3D).

To further characterize the spAsp1 1-pyrophosphatase activity, the kinetic parameters for the hydrolysis of 1-IP7, 5-IP7, and 1,5-IP8 were determined (Table 2). The spAsp1-HAP has a ~12-fold higher turnover (Kcat/|V0|) of 1-IP7 than for 5-IP7, with a ~8-fold difference in catalytic efficiency (Kcat/Km) of 1-IP7 to that of 5-IP7. Additionally, we tested full-length recombinant spAsp1, spAsp1 H397A (HAP pyrophosphatase dead), and spAsp1-D333A (kinase dead mutation) protein for activity toward 1,5-IP8 (Fig. 2E). We observed robust 1-pyrophosphatase activity toward 1,5-IP8 substrate that was dependent on functional histidine catalytic residue (H397).

Comparison of the Catalytic Efficiency of the 1-Kinase and 1-Pyrophosphatase Reactions. Given that the dual activity of Vip1 enzymes produce and destroy 1-PP-IPs at the expense of consuming ATP (a so-called futile cycle), we probed the catalytic efficiencies of the 1-kinase and 1-pyrophosphatase activities of full-length spAsp1 at near-physiological pH (Table 3). This shift in pH caused a threefold increase in the catalytic efficiency of the 1-pyrophosphatase activity observed at pH 5.0. Interestingly, the cause of this drop in catalytic efficiency was a 30-fold increase in the affinity of the enzyme for 1-IP7, offset by a 10-fold decrease in the reaction rate. At pH 7.0, the catalytic efficiency of the 1-pyrophosphatase reaction was 15 times greater than that of the kinase reaction.

We next sought to determine whether the 1-pyrophosphatase is conserved in other species of Vip1 enzymes. Kinetic parameters for the hydrolysis of 1-IP7 by the pyrophosphatase domain of S. cerevisiae Vip1 (scVip1) and by full-length mouse Vip2 (mmVip2) were determined and demonstrate that both species have in vitro PP-IP pyrophosphatase activity (Table 2). A preference for removal of pyrophosphates at the 1-position of PP-IPs was observed with a ~12-fold difference in catalytic efficiency for the mmVip2 hydrolysis of 1-IP7 compared to 5-IP7. The catalytic efficiencies seen for the hydrolysis of 1-IP7, and 1,5-IP8 are similar with Kcat/Km values of 18.6 and 14.0 (×10^3 M^-1 s^-1).

To examine the selectivity of the Vip1 pyrophosphatase activity, we tested synthetically prepared 1-IP7 and 3-IP7, using a polyacrylamide gel electrophoresis (PAGE)-based mass assay. The pyrophosphatase domain of scVip1 readily utilized both enzymatically and synthetically prepared 1-IP7, but did not utilize synthetically prepared 3-IP7 (Fig. 3A). In contrast, hDIPP1 readily utilized both 1-IP7 and 3-IP7 (Fig. 3B), demonstrating a more promiscuous inositol pyrophosphatase activity. Additionally, these data serve as an important control validating the integrity of the synthetic 3-IP7 stereomer. Combination of 1-IP7 and 3-IP7 had no effect on scVip1 pyrophosphatase activity (Fig. 3C). These data demonstrate that the Vip1 class of enzymes are a uniquely selective 1-1-inositol pyrophosphatases.

As mentioned earlier, due to a variety of limitations, our crystallographic analyses of the 1-IP7 or 1,5-IP8 products alone were not sufficient analysis to prove that Vip1 kinase activity exclusively produces D-1 stereomers. With the discovery that Vip1 functions as a selective 1-pyrophosphatase, we performed pyrophosphatase assays on the Vip1 IP7 product. We produced mass quantities of the Vip1 kinase product (Fig. 3A), designated vIP7 and postulated if vIP7 were a mixture of 1- and 3-IP7, then its hydrolysis by 1-pyrophosphatase activity would appear incomplete, leaving residual 3-IP7. Strikingly, we observed complete hydrolysis of vIP7, suggesting that if 3-IP7 is present it is at undetectable levels. Collectively, our structural and enzyme activity/selectivity data demonstrate that Vip1 encodes an evolutionarily conserved class of dual-functional enzyme switches that have 1-kinase activity toward IP7 and 1,5-IP8 substrates and 1-pyrophosphatase activities toward the kinase products 1-IP7, 1,5-IP8, and 1-IP8 (schematically shown in Fig. 3D).

Vip1 Kinase and Pyrophosphatase Activities Regulate Cellular Levels of 1-IP7 and 1,5-IP8. To investigate the relevance of the Vip1 class of kinase and pyrophosphatase activities in cells, we performed a series of high-resolution high-performance liquid chromatography (HPLC) on extracts produced from metabolic labeling of yeast strains expressing various spAsp1 mutants. After steady-state radiolabeling, cell extracts were prepared and separated by chromatography capable of separating individual IP species and, importantly, 5-IP7 and 1-IP7 stereoisomers (confirmed using enzymatically produced standards). Extracts from wild-type cells showed roughly equivalent levels of 5-IP7, 1-IP7, and 1,5-IP8 species; whereas the spAsp1-deficient cells showed a loss of 1-IP7 and 1,5-IP8 and increased 5-IP7 (Fig. 4A, top and second panels). In addition, we examined the effects of overexpression of Asp1 and a series of mutants in wild-type yeast cells (Fig. 4B). Overexpression of full-length spAsp1 lead to an ~10-fold increase in 1-IP7 and 2-fold increase in 1,5-IP8, while the levels of 5-IP7 remaining nearly unchanged (Fig. 4B, top panel). Furthermore, we examined the effects of overexpression of either full-length kinase-dead spAsp1-D333A or a pyrophosphatase domain-only spAsp1-HAP (Fig. 4B, second and third panels), which led to the reduction of 1-IP7 to undetectable levels, and a ~5-10-fold increase in 5-IP7. Overexpression of pyrophosphatase-dead point mutant spAsp1-HAP H397A, did not alter 1P7, and...
IP₈ levels (Fig. 4, bottom panel), confirming metabolic changes observed are attributable to 1-pyrophosphatase activity. Overall, our metabolic data are consistent with Asp1 harboring endogenous 1-kinase and 1-pyrophosphatase activities that are essential for homeostasis of both 1-IP₇ and 1,5-IP₈ in cells.

We next examined the evolutionary conservation of the kinase and pyrophosphatase activities of Vip1 using metabolic studies in human embryonic kidney HEK 293T cell lines genetically engineered to up-regulate PP-IP synthase (18). Of note, our engineered human line dramatically overproduces both IP₇ and IP₈ (Fig. 4, top panel); however, these cells preclude testing pyrophosphatase activity toward 1-IP₇ as its levels are masked by massive amounts of 5-IP₇. Expression of hVip1-HAP reduced 1,5-IP₈ levels alongside a corresponding mass increase in level of 1-IP₇, presumably 5-IP₇ (Fig. 4C, second through fourth panels). The extent of the changes in IP₇ are dependent on the dose of expression level of the pyrophosphatase domain as confirmed by antigen levels in 1×, 3×, and 5× overproducing samples (Fig. 4C and D). Expression of increasing doses of hVip1-HAP(P1400A) catalytic site mutant had no effect on 1,5-IP₈ levels, confirming that decreases in 1,5-IP₈ are dependent on pyrophosphatase domain activity (Fig. 4C, lower two panels, and Fig. 4D).

**Vip1 Kinase and Pyrophosphatase Activities Modulate Vacuolar Morphology.** Previous studies suggest links between spAsp1-deficient mutants and morphological abnormalities including vacuolar size, polarity, and actin cytoskeleton (7, 14, 19). Our results showing that Vip1 enzymes act as a kinase/pyrophosphatase switch provided an impetus to probe the function of each domain and their relative balance in contributing to these phenotypes. We transiently overexpressed a series of vector control, wild type, and spAsp1 mutants and used the acidic dye FM4-64 to examine vacuole size of cells after a 1-h hypotonic shock (Fig. 4E). Hypotonic treatment has previously been shown to activate vacuole fusion in S. pombe through a MAP kinase-dependent signaling pathway (20). Overexpression of full-length spAsp1 resulted in markedly increased average vacuole size and concomitant reduction in the average number per cell compared to vector control (Fig. 4E, top two panels). The diameter of the vacuoles was determined and quantified (Fig. 4F), demonstrating a nearly twofold change in average diameter. We observed significant variation in vacuole size and number within a cell as well as variability among cells within a given population, the latter of which correlated with the intensity of GFP fluorescence signal suggestive of a range of overexpression. These

**Table 2. Kinetic parameters of Asp1/Vip1 pyrophosphatase activity**

| Species   | Construct   | Substrate | $K_m$, μM | $V_{max}$, nmol/min/mg | $k_{cat}$, s⁻¹ | $k_{cat}/K_m$, 10⁸ M⁻¹ s⁻¹ |
|-----------|-------------|-----------|------------|------------------------|----------------|--------------------------|
| S. pombe  | Asp1-HAP    | 1-IP₇     | 17.4       | 227                    | 0.24           | 13.5                     |
| S. pombe  | Asp1-HAP    | 5-IP₈     | 11.4       | 18.3                   | 0.02           | 1.7                      |
| S. pombe  | Asp1-HAP    | 1,5-IP₈   | 14.0       | 123                    | 0.13           | 9.1                      |
| S. pombe  | Asp1-FL-D333A | 1-IP₇     | 15.0       | 93.2                   | 0.16           | 10.9                     |
| S. cerevisiae | Vip1-HAP     | 1-IP₇     | 5.0        | 106                    | 0.12           | 23.4                     |
| M. musculus | Vip2-FL     | 1-IP₇     | 5.9        | 51.2                   | 0.11           | 18.6                     |
| M. musculus | Vip2-FL     | 5-IP₇     | 10.8       | 7.7                    | 0.017          | 1.6                      |
| M. musculus | Vip2-FL     | 1,5-IP₈   | 6.8        | 44.2                   | 0.095          | 14.0                     |

Kinetic constants were determined by fitting enzyme activity determinations of at least three independent measurements by nonlinear regression to the following equation: $Y = V_{max} \times X/(K_m + X)$. 

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**Fig. 2.** spAsp1 is a 1-pyrophosphatase. (A) Schematic of the Vip1 class of dual ATP-GRASP kinase and histidine acid phosphatase domains. Conserved catalytic residues, including aspartic acid and histidine residues in the kinase and His acid phosphatase domains, are noted. (B) spAsp1 acts as a selective 1-IP₇ pyrophosphatase. Reactions were performed with 50 ng of spAsp1-HAP at range of substrate from 2 to 120 μM for Vip1 produced 1-IP₇ and IP6K1 produced 5-IP₇. (C) pH dependence of Asp1-HAP. Twenty micromolar 1-IP₇ was incubated with 25 ng of Asp1-HAP at varying pH values. Each reaction buffer was 100 mM of the indicated buffer, pH = X, 50 mM NaCl. Reaction performed for 15 min at 37 °C. (D) spAsp1 pyrophosphatase activity is dependent on a conserved histidine residue. (Left) Coomassie-stained sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gel of 2 μg each of spAsp1-HAP and spAsp1-FL-HAP-H397A with along molecular weight standards shown in kilodaltons). (Right) Pyrophosphatase assay using fixed substrate 1.25 μM, varying the dose of spAsp1-HAP and spAsp1-FL-H397A. Reaction was performed at pH 5.0 for 15 min at 37 °C. (E) spAsp1 in a 1,5-IP₈ 1-pyrophosphatase. Recombinant full-length spAsp1 wild-type or kinase-dead D333A proteins were incubated with radiolabeled 1,5-IP₈ and robust conversion to 5-IP₇ was observed compared to an H397A catalytic dead mutant.
data are consistent with dose of cellular SpAsp1 as a positive regulator of vacuole fusion and/or inhibitor of fission.

To identify which of SpAsp1’s two domains, if any, and whether or not enzymatic activity is required for the effects observed, we overexpressed a full-length kinase and pyrophosphatase point mutant (SpAsp1 H397A) and full-length kinase-dead pyrophosphatase active point mutant (SpAsp1D333A). Prolonged overexpression of SpAsp1H397A resulted in profound cytotoxicity. Therefore we used an inducible construct from a relatively weak promoter, which prevented cytotoxicity, and found that this construct produced the same vacuole phenotype as overexpression of full-length SpAsp1. A truncation encoding only the kinase domain was lethal when overexpressed in cells, consistent with full-length kinase-only overexpression, whereas expression of full-length SpAsp1 D333A H397A kinase-dead/pyrophosphatase double point mutants produced no vacuolar phenotype. These data, coupled with our metabolic labeling studies, indicate elevation of 1-IP7 and/or 1,5-IP8 is sufficient to induce enlarged vacuoles and the effect is exacerbated by osmotic stress.

To determine whether SpAsp1’s pyrophosphatase domain had any independent effect on vacuole morphology, we expressed a full-length kinase-dead point mutant SpAsp1 D333A and remarkably observed a marked decrease in vacuole diameter alongside an increase in the number of vacuoles (Fig. 4E, third panel). Furthermore, these cells, when osmotically stressed in water, did not undergo any change in vacuole size or number. Cells expressing SpAsp1-HAP or SpAsp1-HAP also displayed defects in the actin cytoskeleton, similar to those previously reported in an asp1 knockout (7, 19).

To elucidate whether this apparent inhibition of vacuole fusion was attributable to SpAsp1’s pyrophosphatase domain alone, a truncation lacking the kinase domain was also expressed. Expression of this pyrophosphatase domain produced a similar phenotype, indicating that the mutant kinase domain is not responsible for these defects. Furthermore, expression of a pyrophosphatase domain catalytically dead point mutant led to normal vacuole morphology, confirming that enzymatic activity is required for the observed phenotype. This also demonstrates a biologically relevant role for SpAsp1 pyrophosphatase domain, its enzymatic activity, and defines key catalytic residues. The distinct vacuolar defects seen with overexpression of the pyrophosphatase domain alone were also rescued by simultaneous expression of the SpAsp1 full-length pyrophosphatase-dead mutant. This result, along with the opposite effects on vacuole fusion seen when independently expressing each activity, suggests that the two SpAsp1 domains have opposing signaling functions.

**Discussion**

In this long-overdue report, we present our historical data, which was not published in a timely manner, confirming that the Vip1 class of inositide metabolic enzymes harbors dual functionality. In addition to a well-documented kinase domain, Vip1 enzymes have an evolutionarily conserved biologically active pyrophosphatase domain. As *Results* is written as though it was a step back in time, it is important to note that several groups have published the existence of Vip1 pyrophosphatase activity and its conservation across the eukaryotic kingdom (13, 16, 21–23). Independently, we made these discoveries (as acknowledged in ref. 23), and importantly, our work provides unequivocal evidence that the Vip1 kinases are exclusive for placing a β-phosphate onto the D-1 phosphomonoester and do not possess observable D-3 kinase activity. This determination emerged from characterization of Vip1 IP7 product through X-ray crystallography in combination with biochemical assays using the Vip1 pyrophosphatase domain. X-ray crystallography of Vip1 products, alone, is not sufficient to make these conclusions (24).

The observed diphosphoinositol phosphohydrolase activity designated 1-pyrophosphatase represents a highly specialized activity for the HAP family of acid phosphatases on substrates other than phosphomonoesters. The HAP domain of Vip1, expressed either alone or in the context of the full-length protein alongside the kinase domain, selectively degrades 1-IP7 and 1,5-IP8, in both in vitro and cellular contexts. This dual-functional nature of Vip1 defines a regulatory homeostatic axis of the inositol phosphate and pyrophosphate metabolic pathway (25).

The tethering of kinase and pyrophosphatase domains on a single peptide that together regulate the synthesis and breakdown of a signaling axis is reminiscent of phosphofructokinase 2 (PFK2) and is a rare example in biology. To our knowledge, the only other known bifunctional futile cycle enzyme with two separate active sites is phosphofructokinase-2/fructose 2,6-bisphosphatase (PFK-2/FBPase-2). In a manner analogous to the Vip1 synthesis and degradation of 1-IP7 and 1,5-IP8, PFK-2/FBPase-2 catalyzes both the synthesis and degradation of fructose 2,6-P2. Fructose 2,6-P2 is a metabolite that allosterically affects the activity of phosphofructokinase 1 (PFK-1) and fructose 1,6-bisphosphatase (FBPase-1) and is a critical modulator of the switch between glycolysis and gluconeogenesis.

We, and others (23), note a number of key elements that enhance pyrophosphatase activity in the HAP domain. First, pyrophosphatase activity was susceptible to expression context: For example, pyrophosphatase activity of GST-mmVIP2 fusion protein was not evident until the GST domain was cleaved from mmVIP1. Additionally, we were unsuccessful in our attempts to express protein harboring pyrophosphatase activity in bacteria for either full length or the pyrophosphatase domains of hVIP1 or hVIP2, even though both proteins exhibited kinase activity, indicating at least partial correct folding of the protein. Rather, recombinant protein made from yeast or mammalian systems harbors pyrophosphatase activity. Of course, in vivo metabolic analysis presented here supports these conclusions. Finally, we note that 1-pyrophosphatase activity in vitro is inhibited by the chloromethylketone class of protease inhibitors, which are routinely used during enzyme purification from cell extracts. The identification of 1-pyrophosphatase activity provides additional mechanistic insight into vacuolar and actin cytoskeletal defects previously seen in *S. pombe* strains either lacking or overexpressing an intact SpAsp1 pyrophosphatase domain. Our further investigation of these *S. pombe* phenotypes here has also revealed a clear correlation between Asp1-regulated 1-IP7 levels and vacuole fusion activity. Modulating relative expression levels of Asp1 pyrophosphatase and kinase activities allows selective accumulation or degradation of 1-IP7, leading to activation or inhibition of vacuole fusion, respectively. It is also notable that osmotic stress, which has previously been linked to elevation of

| Species | Construct | Reaction | $K_{\text{m}}$ μM | $V_{\text{max}}$ mmol/min/mg | $k_{\text{cat}}$ s⁻¹ | $k_{\text{cat}}/K_{\text{m}}$ $10^3$ M⁻¹s⁻¹ |
|--------|----------|---------|----------------|---------------------------|----------------|-----------------|
| *S. pombe* | Asp1-FL | Kinase | 4.6 | 5.8 | 0.010 | 2.2 |
| *S. pombe* | Asp1-FL | Pyrophosphatase | 0.47 | 9.3 | 0.016 | 33 |

Kinetic constants were determined by fitting enzyme activity determinations of at least three independent measurements by nonlinear regression to the following equation: $Y = V_{\text{max}}X/(K_{\text{m}} + X)$. Assays were performed at pH 7.0.
inhibits the C-terminal phosphatase domain (HAP) domains are independent of each other. The KD
the N-terminal kinase domain (KD); whereas the C-terminal histidine acid
pyrophosphatase cycle. We depict Vip1 as a parallel homodimer based on
visualized by toluidine blue staining. (B) Synthetic 3-IP7, utilized as substrate by the nonselective ino-
pyrophosphatase domain with a catalytic point mutation (H397A, and GFP-
D333A constructs were previously described (8). GFP-asp1-HAP, GFP-asp1D333A, and GFP-asp1D397A were subcloned from pREP3X into pREP4X,
and pUN10 using Xhol and BamH restriction sites. To clone the
asp1+ pyrophosphatase domain (asp1-HAP) (residues 377 to 920) and the
asp1+ pyrophosphatase domain with a catalytic point mutation (asp1-
H397A), primers 5′-CTCTACGTCCAAAAGCCGAGGATTATACCACTCGAGCTC
CTAGAAGG-3′ and 5′-CTCTACGTCCAAAAGCCGAGGATTATACCACTCGAGCTC
CTAGAAGG-3′ were used to create an XhoI restriction site (underlined) in
pREP3X-asp1-HAP* and pREP3X-asp1D397A. An XhoI digest was used to
remove the region encoding residues 1 to 376, and the plasmids were reli-
subcloned into pGEX-KG vector from their respective pUN10-asp1 constructs. To confirm the
presence of Vip1 and Asp1 constructs using EcoRI and SalI restriction sites. To clone the
asp1-CTCTAGGCGGAGGATTCAGTCTC
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Materials and Methods
Plasmid Construction. Generation of the pREP3X-GFP- asp1*, asp1D333A, and asp1D397A constructs were previously described (8). GFP-asp1*, GFP-asp1D333A, and GFP-asp1D397A were subcloned from pREP3X into pREP4X, pREP4X, and pUN10 using Xhol and BamH restriction sites. To clone the
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asp1+ pyrophosphatase domain with a catalytic point mutation (asp1-
H397A), primers 5′-CTCTACGTCCAAAAGCCGAGGATTATACCACTCGAGCTC
CTAGAAGG-3′ and 5′-CTCTACGTCCAAAAGCCGAGGATTATACCACTCGAGCTC
CTAGAAGG-3′ were used to create an XhoI restriction site (underlined) in
pREP3X-asp1-HAP* and pREP3X-asp1D397A. An XhoI digest was used to
remove the region encoding residues 1 to 376, and the plasmids were reli-
subcloned into pGEX-KG vector from their respective pUN10-asp1 constructs. To confirm the
presence of Vip1 and Asp1 constructs using EcoRI and SalI restriction sites. To clone the
asp1-CTCTAGGCGGAGGATTCAGTCTC
GAAGATTCAGTCTC
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A pET-28-based expression plasmid (pNIC-Bsa4) encoding human diphosphoinositol polyphosphate phosphohydrolase 1 (Dipp1) (Nudt3; accession number AAH07727) residues 1–148 was kindly provided by the Structural Genomics Consortium, Karolinska Institutet (Stockholm, Sweden).

**Recombinant Protein Expression and Purification.** Constructs of *S. pombe* asp1-HAP, asp1D333A, *S. cerevisiae* Vip1-HAP, and *Mus musculus* Vip2 were transformed and expressed as GST-fusion proteins in *Escherichia coli*. *S. pombe* and *S. cerevisiae* constructs were expressed in BL21(DE3) cells by growing cultures initially at 37 °C, and then reducing temperature to 18 °C at OD600 of 0.6 for a 16-h induction with a final concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The murine Vip2-FL construct was expressed in ArcticExpress (DE3)RIL cells by growing cultures at 30 °C initially, and then reducing to 12 °C. The cells were induced at OD600 of 0.6 for 24 h with a final concentration of 0.25 mM IPTG.

Cells were collected by centrifugation and resuspended in lysis buffer containing 25 mM Tris, pH 8.0, 350 mM NaCl, 2 mM DTT, and 0.1 mM β-mercaptoethanol.

**Table 4. Strains used in this study**

| *S. pombe* strain | Genotype | Source |
|-------------------|----------|--------|
| JYY889            | JYY841 + pREP3X | Ref. 7 |
| JYY889            | JYY841 + pREP3X-GFP-Asp1 | Ref. 7 |
| JYY903            | JYY841 + pREP3X-GFP-asp1D333A | Ref. 7 |
| JYY905            | JYY841 + pREP3X-GFP-asp1H397A | Ref. 7 |
| JYY1083           | JYY841 + pREP3X-GFP-asp1-HAP | This work |
| JYY1097           | JYY841 + pREP3X-GFP-asp1-HAPH397A | This work |
| JYY1077           | JYY841 + pREP3X-GFP-asp1H397A | This work |
| JYY1078           | JYY841 + pREP3X-GFP-asp1H397A | This work |
| JYY845            | JYY841 + pREP3X-GFP-asp1D333A + pREP41X | This work |
| JYY847            | JYY841 + pREP3X | This work |

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phenylmethanesulphonyl fluoride (PMSF). Cell resuspensions were lysed by serial passage through an EmulsiFlex-C5 high-pressure homogenizer at 15,000 psi. Lysis debris was cleared by centrifugation, and the resulting paste was resuspended in 20% (wt/vol) lysis buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 100 mM imidazole, and 2 mM DTT). The Ni-NTA protein fractions were pooled, concentrated, and applied to a S-200 size exclusion column. The purified protein in 20 mM Hepes, pH 7.5, 150 mM NaCl, and 2 mM DTT was quantified by UV absorbance under denaturing condition and stored at −80 °C.

Hexa-histidine–tagged human Dipp1 residues 1 to 148 were produced by transformation and overexpression of the pNIC-Bsa-hDipp1 plasmid in BL21(DE3) cells similar to previously described (35). Cultures were grown at 37 °C to an OD of 0.5, after which the temperature was reduced to 18 °C and the cultures were induced with IPTG to a final concentration of 0.5 mM. After overnight induction, the cells were collected by centrifugation, and the resulting paste was resuspended in 20% (v/vol) lysis buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 100 mM imidazole, and 2 mM DTT). The Ni-NTA and PMSF were added and lysed by multiple passes at ~15,000 psi through an EmulsiFlex-C5 high-pressure homogenizer. The resulting lysate was clarified by centrifugation and applied to Ni-NTA agarose affinity column (Qiagen) in equilibrated in lysis buffer. The column was washed with 20 column volumes lysis buffer, and the protein was step eluted in 50 mM Hepes, pH 7.5, 500 mM NaCl, 100 mM imidazole, and 2 mM DTT. The Ni-NTA protein fractions were pooled, concentrated, and applied to a Vivaspin 20 (10,000 MWCO) and injected onto a S-200 size exclusion column (GE Biosciences) equilibrated with 20 mM Hepes, pH 7.5, 300 mM NaCl, 10% glycerol, and 0.5 mM tris(2-carboxyethyl) phosphate. The Dipp1-containing 200 fractions were pooled and concentrated to 12 mg/mL as determined by UV absorbance, V20, 19,643 1/M cm, under denaturing conditions. Small aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Preparation and Purification of Mass Quantities of Biological and Synthetic PP-IPs. Biological isomers of IP5 and IP6 were enzymatically produced as previously described (7, 8). Labeled synthetic isomers of IP7 and IP8 were obtained from the laboratory of G. Prestwich (University of Utah, Salt Lake City, UT) and either VIP1/Asp1 or Dipp protein at varying dosage. Enzyme assays were run 20 to 40 min at 37 °C and quenched by boiling for 5 min. After centrifugation, 6× loading dye (10 mM Tris, pH 6.8, 1 mM EDTA, 30% glycerol, and 0.25% bromophenol blue) was added to the supernatant and the samples were run at 300 V for ∼2.5 h on a 33.3% TBE-PAGE gel. The gels were stained and destained for visualization as described (42).

Fission Yeast Growth and Manipulation. S. pombe strains were manipulated, propagated, and transformed using published standard procedures (43) and as described previously (7). For lithium acetate transformation of expression plasmids, 1 μg of DNA was used along with ~1 × 108 log-phase cells as described (43), and the mixture was then plated onto MMA agar plate supplemented with 5 mg/L thiamine (Sigma Chemical Company) and 225 mg/L of polyethyleneimine cellulose TLC plates (JT Baker) and resolved in a tank equilibrated in 2.10 M HCl, 1.09 M KH2PO4, and 0.72 M K2HPO4. TLC plates were dried, exposed to a phosphor storage screen, and quantified using a 4500 SI PhosphorImager (Amersham Biosciences).

PAGE analysis using toluidine blue staining was used to visualize the pyrophosphatase activity of Dipp1 and VIP1/Asp1 against unlabeled biological and synthetic IP7 isomers (42). Ten-microliter reactions containing 1 μM of PP-IP7 substrate at various pH values, 50 mM NaCl, and either VIP1/Asp1 or Dipp protein at varying dosage. Enzyme assays were run 20 to 40 min at 37 °C and quenched by boiling for 5 min. After centrifugation, 6× loading dye (10 mM Tris, pH 6.8, 1 mM EDTA, 30% glycerol, and 0.25% bromophenol blue) was added to the supernatant and the samples were run at 300 V for ~2.5 h on a 33.3% TBE-PAGE gel. The gels were stained and destained for visualization as described (42).
enzymatically produced [32P]-IP7 and [32P]-IP8 standards. HEK 293T cells were radiolabeled and harvested as previously described (8). Inositol phosphates from these extracts were analyzed on a 4.6 x 125-mm Partisphere SAX HPLC column (Whatman) as previously reported (8). Experiments were performed using a minimum of three independent samples and representative HPLC traces are shown. y axis indicated relative counts (cpm) normalized to each sample's total radioactivity.

**Fission Yeast Vacuole Microscopy.** The membrane-selective dye FM 4-64 (Invitrogen) was used for vacuole staining of the S. pombe strains. For each strain, 400 μL of log-phase cells were pelleted by low-speed centrifugation and resuspended in yeast extracts with supplements (YES medium) plus FM 4-64 at a concentration of 16 μM. After a 30-min incubation at 30 °C, 1 mL of YES was added, and cells were cultured for an additional 30 min. For vacuole fusion analysis, FM 4-64-stained cells were washed to remove media and placed in water. Cells were visualized after 1-h incubation in water to measure vacuole diameter during hypotonic shock. Digital images were obtained using a Nikon Eclipse TE 2000-E microscope equipped with a 40x objective. The vacuole diameter measurements (in microns) were made with the Nikon Elements Analysis software package. Data were plotted using Prism software, and statistics were performed using ANOVA Tukey's multiple-comparison test.

**Data Availability Statement.** All materials, methods, and data are freely available upon request from the J.D.Y. laboratory. Structural data have been deposited in the PDB under ID codes 6PCL and 6PCK.

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