The Vip1 Inositol Polyphosphate Kinase Family Regulates Polarized Growth and Modulates the Microtubule Cytoskeleton in Fungi

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Abstract

Microtubules (MTs) are pivotal for numerous eukaryotic processes ranging from cellular morphogenesis, chromosome segregation to intracellular transport. Execution of these tasks requires intricate regulation of MT dynamics. Here, we identify a new regulator of the Schizosaccharomyces pombe MT cytoskeleton: Asp1, a member of the highly conserved Vip1 inositol polyphosphate kinase family. Inositol pyrophosphates generated by Asp1 modulate MT dynamic parameters independent of the central +TIP EB1 and in a dose-dependent and cellular-context-dependent manner. Importantly, our analysis of the in vitro kinase activities of various S. pombe Asp1 variants demonstrated that the C-terminal phosphatase-like domain of the dual domain Vip1 protein negatively affects the inositol pyrophosphate output of the N-terminal kinase domain. These data suggest that the former domain has phosphatase activity. Remarkably, Vip1 regulation of the MT cytoskeleton is a conserved feature, as Vip1-like proteins of the filamentous ascomycete Aspergillus nidulans and the distantly related pathogenic basidiomycete Ustilago maydis also affect the MT cytoskeleton in these organisms. Consistent with the role of interphase MTs in growth zone selection/maintenance, all 3 fungal systems show aspects of aberrant cell morphogenesis. Thus, for the first time we have identified a conserved biological process for inositol pyrophosphates.

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Introduction

Cell polarization can be viewed as the generation and upkeep of a defined cellular organization. The readout of cell polarization in fungal systems is polarized growth resulting in a specific cell shape and size. This ranges from the 14 μm long cylindrical Schizosaccharomyces pombe fission yeast cell, which maintains its shape by restricting growth zones in a cell cycle dependent manner to the extremely polarized growth of filamentous fungi such as Aspergillus nidulans where hyphal extension can occur in a continuous and infinite manner [1–3]. Fungi are capable of morphological transitions in response to external signals and this represents an important virulence trait of pathogenic fungi such as the corn smut fungus Ustilago maydis. Here, the transition from a non-pathogenic haploid yeast-like form to a dikaryotic filament is required for the fungus to enter the host tissue [4]. Such an alteration in growth form is also present in non-pathogenic model yeasts such as S. cerevisiae and S. pombe where it acts as a foraging response [5–7]. Polarized growth in fungi depends on the interplay between the MT and actin cytoskeletons and in some systems septins [8]. In S. pombe, where growth occurs at the cell tips which contain oscillating Cdc42, actin cables are used for the transport of growth vesicles. On the other hand, MT plus-end dependent transport of the landmark complex Tea1-4 via the kinesin Tea2 is required for marking potential zones of growth [1,9–13]. Correct delivery of Tea1-4 requires alignment of antiparallel interphase MTs along the long axis of the fission yeast cell. The dynamic MT plus-ends are oriented and polymerize towards the cell end; upon contact with the tip MT dynamics are modified, the landmark complex unloaded and anchored at the cell tip [14–16]. MT dynamics are regulated mainly by the diverse group of proteins at the MT plus-end. Here, the central component is the conserved EB1 family, which is essential for plus-end association of numerous +TIPs [17]. Interestingly, the Tea1-4 complex is also present in filamentous fungi where a recent publication has uncovered additional functions namely regulating MT dynamics and MT
Fungi are an extremely successful and diverse group of organisms ranging from the small single-celled yeasts to the indefinitely growing filamentous fungi. Polarized growth, where growth is restricted to defined regions, leads to the specific cell shape of yeast cells, as well as the very long hyphae of filamentous fungi. Fungal polar growth is controlled by an internal regulatory circuit of which the microtubule cytoskeleton comprises the transport road for numerous cargos needed for polarized growth. However, the microtubule cytoskeleton is not static, but a dynamic structure, which is modulated by microtubule-associated proteins and the interaction with other cellular structures. Our present analysis has identified a new regulator of the microtubule cytoskeleton in the fission yeast S. pombe: a member of the highly conserved Vip1 inositol polyphosphate kinase family. Vip1 proteins have a dual domain structure consisting of an N-terminal kinase domain which synthesizes inositol pyrophosphates and a C-terminal domain, which we show to negatively regulate the kinase output. Our results suggest that modulation of microtubule dynamics is correlated to Vip1 kinase activity. Importantly, polarized growth and microtubule dynamics were also modulated by Vip1 family members in A. nidulans and U. maydis thus uncovering a conserved biological role for inositol pyrophosphates.

Inositol pyrophosphates regulate cellular processes by two different modes of action: (i) modulation of protein function by reversible binding of these high energy molecules and (ii) protein phosphorylation [50,51]. An example for the first type of regulation is the Akt kinase which is involved in insulin signaling. Here, specific inositol pyrophosphates were shown to bind to the Akt PH domain thus blocking activation of this kinase [52]. An example for the second type of action is the regulation of the antiviral response via activation of the interferon transduction factor IRF3. In a cell free system IRF3 was phosphorylated by specific inositol pyrophosphates and this process required the transfer of the β-phosphate of the pyrophosphate group [49].

The cellular processes regulated by inositol pyrophosphates are wide-ranging and diverse. These include the phosphate availability response in S. cerevisiae, the chemotactic response in Dictyostelium, the antiviral response and insulin signaling in mammals and the dimorphic switch in S. pombe [7,49,50,52,53]. We have now extended our analysis of Vip1 biological function and have found that inositol pyrophosphates have a conserved role in fungal morphogenesis.

**Results**

**The Vip1 family member Asp1 generates IP₇ in vitro**

We had previously generated S. pombe strains that expressed the endogenous Asp1 variants Asp₁¹⁸⁵₃₃A and Asp₁³⁹₇₉₇A [7]. The former Asp1 variant has a single amino acid change at position 333, a key catalytic residue for kinase activity, while H397 is a highly conserved histidine residue of the C-terminal acid phosphatase-like domain (Figure 1A) [43]. Phenotypic analysis of these Asp1 variant expressing strains suggested that Asp₁¹⁸⁵₃₃A and Asp₁³⁹₇₉₇A have an altered enzymatic activity compared to the wild-type Asp1 protein [7]. Therefore we assayed the ability of Asp₁¹⁸⁵₃₃A and Asp₁³⁹₇₉₇A to generate inositol pyrophosphates.

As it had not been demonstrated previously that the S. pombe Asp1 protein could generate inositol pyrophosphates, we first tested with an in vitro assay if this was the case. Asp1 was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein and purified. Using a recently published method that allows analysis of inositol pyrophosphates by PAGE, we found that purified GST-Asp1 generated inositol pyrophosphates (from now on called IP₇) in an ATP-dependent manner using IP₆ as a substrate (Figure S1A, right panel) [54]. This activity was dose-dependent, as the amount of IP₇ generated increased with increasing amounts of protein used (Figure S1B). We next tested
if the GST-tagged Asp1^{D333A} and Asp1^{H397A} proteins (Figure 1B) also generated IP_{7} and found that Asp1^{D333A} was unable to convert IP_{6} to IP_{7} (Figure 1C, lane 5). Interestingly, comparing equal amounts of protein, the Asp1^{H397A} variant generated more IP_{7} than the wild-type Asp1 protein (Figure 1C, lane 6 and 4, respectively). Analysis of IP_{7} production by Asp1 and Asp1^{H397A} proteins over a time period of 0 to 10 hrs revealed that IP_{7} production increased with time and that Asp1^{H397A} could produce up to 100% more IP_{7} than the wild-type Asp1 protein (Figure 1D, left panel, lanes 7–12 and 1–6, respectively; quantification shown in 1E). Similar results were obtained when comparing IP_{7} production of the wild-type Asp1 and the Asp1^{1-364} variant.
(contains only the kinase domain) (Figure S12). These data point to a negative role of the C-terminal acid phosphatase-like domain. To analyze this further we determined the \( K_m \) and \( V_{\text{max}} \) values for Asp1 and Asp1\(^{H397A} \) (Figure 1F). The \( K_m \) values for Asp1 and Asp1\(^{H397A} \) were 58.18 \( \mu M \) and 57.32 \( \mu M \) respectively implying a similar affinity for the substrate. However the \( V_{\text{max}} \) for Asp1\(^{H397A} \) was 36% higher than that of Asp1 (Figure 1F).

To learn more about the negative impact of the Asp1 phosphatase-like domain on IP\(_7\) production, we (i) tested if addition of Asp1\(^{365-920} \) reduced the inositol pyrophosphate out-put in an Asp1 \textit{in vitro} kinase assay and (ii) determined the \textit{in vivo} read-out of Asp1 variants with mutations in conserved residues of the phosphatase-like domain (Figure 2A).

The presence of purified bacterially expressed GST-tagged Asp1\(^{365-920} \) in the IP\(_7\) \textit{in vitro} assay together with full length Asp1 reduced the amount of IP\(_7\) in a dose-dependent manner (Figure 2B). IP\(_6\) amounts were unaffected by Asp1\(^{365-920} \) as shown by the incubation of only this Asp1 variant with IP\(_6\) in the \textit{in vitro} assay (Figure S13). Thus, the negative effect was only seen for the IP\(_7\) output. We therefore propose that the Asp1 C-terminal
phosphatase-like domain has phosphatase activity and its substrate is inositol pyrophosphate generated by the Asp1 N-terminal kinase domain (see discussion).

We had shown previously that the \textit{asp1}^{H397A} strain was more resistant to microtubule poisons such as thiabendazole (TBZ) while the \textit{asp1}^{D333A} strain was more sensitive to TBZ compared to the wild-type strain [7]. A deletion of \textit{asp1} (\textit{asp1}\textsuperscript{−}\textit{−}) strain also led to TBZ hypersensitivity (Figure S2A). Furthermore a strain where the wild-type \textit{asp1}\textsuperscript{+} had been replaced by the \textit{asp1}^{D333A, H397A} strain also showed the same increased TBZ sensitivity as the \textit{asp1}^{D333A} and \textit{asp1}\textsuperscript{Δ} strains (Figure S2A). These data strongly suggest that the TBZ resistance/sensitivity of these strains is solely dependent on the function of the Asp1 kinase. Absence of Asp1 kinase activity results in TBZ hypersensitivity (\textit{asp1}\textsuperscript{−}, \textit{asp1}^{D333A} and \textit{asp1}^{D333A, H397A} strains) while increased Asp1 kinase function (\textit{asp1}^{H397A} strain) results in TBZ resistance. The Asp1 C-terminal phosphatase-like domain appears to modulate only the function of the Asp1 N-terminal kinase domain as the \textit{asp1}^{D333A, H397A} strain has the same TBZ phenotype as \textit{asp1}^{+} and \textit{asp1}^{D333A} strains (Figure S2A).

These results demonstrate that increased TBZ resistance can be used as an \textit{in vivo} read-out for a non-functional Asp1 phosphatase domain. We expressed wild-type \textit{asp1}\textsuperscript{+} and the mutant versions \textit{asp1}^{H397A}, \textit{asp1}^{H397A, D333A} and \textit{asp1}^{1-794} on a plasmid from the thiamine-repressible \textit{nmt1} promoter [53] in the \textit{asp1}\textsuperscript{Δ} strain. Western blot analysis revealed that expression of full length Asp1 variants was similar (Figure S14). Expression of these \textit{asp1} variants except \textit{asp1}^{H397A} did not affect cell growth (Figure 2C, growth on TBZ containing plates). Plasmid-borne high expression of \textit{asp1}^{H397A} is lethal as has been shown previously [43].

As shown in Figure 2C plasmid-borne expression of full length \textit{asp1}\textsuperscript{+} allowed partial growth of the \textit{asp1}\textsuperscript{Δ} strain on TBZ containing plates. However expression of \textit{asp1}^{1-794} and \textit{asp1}^{D333A} resulted in better growth of the \textit{asp1}\textsuperscript{Δ} strain on TBZ medium. We conclude that the conserved phosphatase signature motif is required for the function of the C-terminal domain.

To test if the Asp1 C-terminal domain is also able to regulate Asp1 kinase activity \textit{in trans in vivo}, we constructed a plasmid, which expressed \textit{asp1}^{1-364} and \textit{asp1}^{365-920} from two separate \textit{nmt1} promoters on the same plasmid (Figure S3A). Expression of this plasmid in the \textit{asp1}\textsuperscript{Δ} strain resulted in a similar phenotype as \textit{asp1}\textsuperscript{+} (Figure 2D, protein levels shown in Figure S3B–C). Thus in this \textit{in vivo} situation, it appears that both Asp1 domains need to be on the same molecule for the negative impact of the C-terminal domain to be exerted.

\textbf{Asp1 affects interphase MT organization}

Our \textit{in vitro} kinase assay demonstrated that the \textit{asp1}^{D333A} variant has no enzymatic activity, while that of \textit{asp1}^{H397A} is higher than that of the wild-type Asp1 protein. Thus it is very likely that the resistance/sensitivity to microtubule poisons is a result of different intracellular inositol pyrophosphate levels.

We had previously identified a truncated Asp1 variant (\textit{asp1}^{1-794}) as a multi-copy suppressor of the TBZ-hypersensitivity of a \textit{mal3} mutant strain [7]. \textit{Mal3} is the fission yeast member of the EB1 family of MT associated proteins [56]. We therefore determined if Asp1 function modulated the MT cytoskeleton by analyzing the interphase MT cytoskeleton of the various \textit{asp1} strains via expression of GFP-\zeta-tubulin (using the endogenous \textit{nmt81}:\textit{gfp-atb2} construct) [57]. \textit{asp1} variant strains with or without the presence of GFP-\zeta-tubulin had a similar growth phenotype (Figure S4A).

In \textit{S. pombe}, interphase MTs are polymerized in the vicinity of the nucleus, align along the long axis of the cell and grow with their plus-ends towards the cell end, where they pause prior to depolymerization [50]. The organization of interphase MTs of the \textit{gfp-atb2} \textit{asp1}^{H397A} strain was comparable to the wild-type strain while those of the fainter fluorescent \textit{gfp-atb2} \textit{asp1}^{D333A} and \textit{gfp-atb2} \textit{asp1}\textsuperscript{Δ} MTs appeared to be more disorganized (Figure 3A). In particular, the number of interphase MTs that depolymerized at the lateral cortex/cytoplasm and not at the cell tip was increased significantly in \textit{asp1}^{D333A} and \textit{asp1}\textsuperscript{Δ} cells compared to wild-type cells (Figure 3B). An example is shown for an \textit{asp1}^{D333A} MT that touched the lateral cortex and became depolymerized instead of being deflected as seen for such MTs in \textit{asp1}\textsuperscript{−} cells (Figure S4B).

\textbf{Inositol pyrophosphates regulate interphase MT dynamics}

Measurement of MT parameters in the 3 \textit{asp1} variant strains revealed that MT dynamics were altered. \textit{asp1}^{D333A} cells showed an increased MT growth rate while the rate of MT shrinkage was decreased in \textit{asp1}^{H397A} cells (Table 1). Interphase MTs of \textit{asp1}^{D333A} cells had an increased number of catastrophe events while those of \textit{asp1}^{H397A} cells were reduced compared to wild-type cells (Table 1). The average MT length for both \textit{asp1} mutant strains was increased compared to the MTs of the wild-type strain. Thus, all measured MT parameters were affected by intracellular inositol pyrophosphate levels. \textit{asp1}^{D333A} MTs are more dynamic, whereas \textit{asp1}^{H397A} MTs have the opposite phenotype.

Interestingly, we found that the residence time of the MT plus-end at the cell tip was dependent on the \textit{asp1} variant expressed in the cell. Measurement of the time that a MT stays at the cell tip showed that the residence time of a MT plus-end at the cell tip is variable. Nevertheless, when we compared this MT parameter for wild-type and \textit{asp1}^{D333A} cells we found that the latter MTs had on average a significantly reduced pausing time at the cell tip before depolymerization (Table 1). For example, only 12% of \textit{asp1}^{D333A} MTs but 28% of wild-type MTs paused at the cell tip for more than 60 seconds (Figure 3C). In contrast, the residence time of \textit{asp1}^{H397A} MT plus-ends at the cell tip appeared to be increased compared to wild-type MTs but this was not statistically significant (Table 1). We therefore increased Asp1 generated inositol pyrophosphate levels even further by plasmid-borne expression of the \textit{asp1} variant \textit{asp1}^{1-364} (kinase only) in the \textit{asp1}^{H397A} strain. Under these conditions the average MT pausing time was increased by 30% in cells expressing \textit{asp1}^{1-364} (\textit{asp1}^{H397A} strain plus vector: 42.2±18.6 seconds; \textit{n}=105; \textit{asp1}^{H397A} strain plus \textit{asp1}^{1-364}: 53.6±38.4 seconds; \textit{n}=110; \textit{p}<0.025 (t-test)). A detailed depiction of MT pausing time in this assay is shown in Figure 3D and an example of the increased MT residence at the cell tip is shown in Figure 3E.

Thus, inositol pyrophosphate levels appear to regulate the residence time of a MT plus-end at the cell tip. Increasing the levels of Asp1 generated inositol pyrophosphates increases pausing at the tip prior to a catastrophe event, while lowering the amount of Asp1 generated inositol pyrophosphates has the opposite effect.

\textbf{Asp1 regulated MT dynamics occur independently of the +TIP protein Mal3}

Proteins associated with MT plus-ends play a leading role in regulating MT dynamics [59]. Of particular importance is the...
Figure 3. Asp1 kinase function affects MT organization. (A) Live cell images of the indicated strains expressing nmt81::gfp-atb2. The same imaging and image-processing conditions were used for all strains. Bar, 5 μm. (B) Percentage of MTs depolymerizing at a cell end or at the lateral cortex/cytoplasm. Wild-type: n = 102, asp1H397A: n = 218, asp1D333A: n = 166, asp1Δ: n = 131. ** P < 0.005 for asp1D333A or asp1Δ compared to wild-type as determined using χ²-test. (C) MT pausing time (sec) at cell ends in the indicated strains. Overall MT pausing time of these strains is shown in Table 1. We arbitrarily defined the 4 categories to show the variability within this system. Wild-type: n = 100, asp1H397A: n = 67, asp1D333A: n = 75. (D) MT pausing time (sec) at cell ends in the asp1H397A strain transformed with a vector control or expressing pasp11-364. Overall MT pausing time of these strains is shown in Table 1. Cells were grown in plasmid-selective minimal medium. asp1H397A + vector: n = 105, asp1H397A + pasp11-364: n = 110. pasp11-364 denotes plasmid-borne expression of Asp11-364 via the nmt1 promoter under promoter de-repressing conditions. (E) Live cell images of
EB1 family, which is central to the association of other +TIPs with the MT plus-end. To determine if Asp1 affects MT dynamics via the EB1 family member Mal3, double mutant strains between mal3Δ (mal3 deletion) and the asp1Δ alleles asp1H397A, asp1D333A and asp1Δ were constructed. The asp1H397A mal3Δ strain showed a reduced TBZ sensitivity compared to the single mutant mal3Δ strain, demonstrating that increased Asp1 kinase function rescues the mal3Δ mutant TBZ phenotype (Figure 4A). Loss of Asp1 kinase activity increased the TBZ hypersensitivity of mal3Δ strains as shown for the asp1D333A mal3Δ and asp1Δ mal3Δ strains (Figure 4A). Similar results were obtained when these strains grew on medium containing the MT drug methyl-benzimidazol-2-yl-carbamate (MBC) (Figure 4B). We next assayed if plasmid borne overexpression of the Asp1 variant Asp1H397A (Asp1 kinase domain only), rescued the mal3Δ TBZ hypersensitivity, and found this to be the case (Figure 4C). Furthermore increasing intracellular IP7 levels by other means than asp1+ manipulation, namely by using a strain where the ORF coding for the nudix hydrolase Aps1 was deleted (asp1Δ), also decreased the TBZ hypersensitivity of the mal3Δ strain (Figure 4D). Nudix hydrolases degrade inositol pyrophosphates and disruption of the nudix hydrolase encoding gene increases the intracellular concentration of inositol pyrophosphates 3-fold [60,61].

As Mal3 stabilizes MTs, mal3Δ cells do not have a normal interphase MT-cytoskeleton, where MTs are aligned in antiparallel3 budding order. This short interphase MT phenotype was rescued partially in the mal3Δ asp1H397A strain (Figure 4E). We determined MT parameters in the mal3Δ asp1H397A strain (Figure 4F). MTs grew longer before a catastrophe event in the mal3Δ asp1H397A strain compared to the single mutant mal3Δ strain (Table 2 and Figure 4F). Thus MT dynamics regulation by inositol pyrophosphates does not require the EB1 protein.

Next, we analyzed Mal3-GFP particle movement in the various asp1+ strains. The EB1 family decorates MTs and forms the comet-shaped structures at the MT plus-end characteristic of plus-end tracking proteins [59,63]. The Mal3-GFP distribution on MTs was similar in all asp1+ strains and was as described [63]. We determined the speed of the outmost outbound Mal3-GFP comets moving towards the cell end. As shown in Figure 4G movement of such Mal3-GFP particles in the wild-type and asp1H397A strain was similar, while asp1D333A Mal3-GFP comets were faster. The speed of movement of outmost outbound Mal3-GFP was directly correlated to the MT growth rate of the particular asp1 strain (Table 1 and Figure 4G). We also assayed movement of the kinesin Tea2-GFP in the 3 asp1+ variant strains and found that the speed of Tea2-GFP signals at the end of MTs was comparable to Mal3-GFP comets (Figure S17).

Asp1 kinase function is required for growth zone selection in S. pombe

Interphase MTs in S. pombe control proper polarized growth by delivering the Tea1-4 landmark protein complex to potential sites of growth at the cell tip [10,12,64,65]. Consequently, an aberrant interphase MT cytoskeleton can result in an altered positioning of the growth zones and in cells with a branched or bent morphology.

In wild-type fission yeast cells growth at a specific cell end is cell cycle controlled. After cytokinesis, cells will first grow in a monopolar manner selecting the old end (the end present before the previous cell division) as the first growth zone. The attainment of a critical cell size and completion of S-phase allow a switch to bipolar growth (NETO transition) at both cell ends in the G2 cell cycle phase [66,67]. We had shown previously that Asp1 kinase function is essential for NETO, as 84% of asp1D333A cells grow exclusively monopolar on an agar surface using the old end as the site of growth [7]. However, the cylindrical cell shape was maintained in most asp1D333A cells demonstrating that the growth zone was still at the cell end. Abnormal growth zone positioning i.e. the selection of a growth zone not at the cell tip was observed in less than 5% of asp1D333A cells [7].

Next we asked, if proper polarized growth could also be re-established in asp1+ mutants that were re-entering the vegetative cell cycle after nutrient starvation. Re-entry of cells into the cell cycle requires the presence of a MT minus-end, the presence of which is controlled by the MT plus-end tracking proteins (MT+TIPs). Inositol pyrophosphates have been shown to be involved in MT catastrophe (sec) length (Table 1 and Figure 4G). We also assayed movement of the kinesin Tea2-GFP in the 3 asp1+ variant strains and found that the speed of Tea2-GFP signals at the end of MTs was comparable to Mal3-GFP comets (Figure S17).

**Table 1.** Interphase MT dynamics in asp1+ variant strains.

| strain | growth (nm/sec) | rate of shrinkage (nm/sec) | growth before catastrophe (sec) | length (μm) | pausing at tips (sec) |
|--------|-----------------|---------------------------|-------------------------------|-------------|----------------------|
| wild-type | 56.8±26.5 (n=90) | 154.8±66.5 (n=72) | 81.3±32.3 (n=100) | 6.3±1.2 (n=100) | 47.3±38.0 (n=100) |
| asp1D333A | 66.7±27.2*↑ (n=90) | 145.6±67.5 (n=45) | 68.3±29.4↓ (n=126) | 6.9±1.1*↑ (n=86) | 34.5±29.1*↓ (n=75) |
| asp1H397A | 58.2±20.9 (n=90) | 126.7±50.2↓ (n=92) | 99.0±39.2↑ (n=155) | 7.3±1.4*↑ (n=100) | 50.7±35.7 (n=67) |

Parameters of MT dynamics were measured for the indicated strains expressing nmt81::gfp-atb2+. Cells were grown in non-selective minimal medium, n, number of MTs measured; asterisks denote significance between wild-type and mutant, + or −, parameter is decreased or increased compared to wild-type, growth: * p<0.01 for asp1D333A vs. wild-type (t-test). Rate of shrinkage: *p<0.005 for asp1H397A vs. wild-type (t-test). Length: *p<0.0005 for asp1D333A vs. wild-type (Welch-test). Pausing at tips: *p<0.01 for asp1D333A vs. wild-type (Welch-test).
cycle from G0 requires a *de novo* definition of the growth zones. [13,16]. We thus examined the morphology of *asp1*Δ and *asp1* mutant cells after exit from stationary phase: on agar 93% of *asp1*Δ, 100% of *asp1*ΔΔ and 73% of *asp1*ΔΔΔ cells grew as normal cylindrically shaped cells (Figure 5A). The remaining 27% of growing cells had an abnormal morphology, indicating that proper polarized growth was not re-established (Figure 5A–B). Incubation of stationary *asp1*ΔΔΔ cells into fresh liquid medium...
massively aggravated the ectopic growth phenotype: under these conditions 80% of the cells had an aberrant, branched or lemon-shaped appearance indicating that Asp1 kinase activity was required for polarized growth and growth zone selection under these circumstances (Figure 5C–D). We also determined if cells when exiting from G0 state on solid medium showed the monopolar to bipolar growth pattern of exponentially growing cells. However we found a wide variety of growth patterns even for

### Table 2. Interphase MT dynamics in mal3Δ variant strains.

| strain         | growth (nm/sec) | length (μm) |
|----------------|-----------------|--------------|
| mal3Δ          | 37±23 (n = 90)  | 2.1±0.3 (n = 54) |
| mal3Δ asp1H397A| 54±35* (n = 91) | 2.7±0.6* (n = 53) |

Parameters of MT dynamics were measured for the indicated strains expressing nmt81::gfp-atb2+. Cells were grown in non-selective minimal medium. n, number of MTs measured; asterisks denote significance between mal3Δ and mal3Δ asp1H397A; ↑, parameter is increased compared to mal3Δ. Growth: * p < 0.0005 for mal3Δ asp1H397A vs. mal3Δ (t-test). Length: * p < 0.0005 for mal3Δ asp1H397A vs. mal3Δ (t-test). doi:10.1371/journal.pgen.1004586.t002

![Figure 5. Asp1 is required for growth zone selection.](image-url)
the \textit{asp1}^+ cells indicating that cells need to undergo a number of cell divisions before the normal growth pattern is stably re-established. It was thus not possible to determine if \textit{asp1}^{D333A} cells deviate from the norm.

\textit{A. nidulans} Vip1-like protein is required for polarized growth

As the Vip1 family is conserved from yeast to man, we determined if Vip1 members also played a role in cell morphogenesis in other organisms. We therefore analyzed the function of Asp1-homologues in the filamentous ascomycete \textit{Aspergillus nidulans} and the dimorphic basidiomycete \textit{Ustilago maydis}. In both fungi, the importance of the MT cytoskeleton for fungal growth has been investigated extensively \cite{18,21,26,31,68,69}. We decided to generate and characterize strains where the genes coding for the Asp1-homologues had been deleted as we have shown for \textit{S. pombe} that the \textit{asp1}^\Delta strain behaved identical to the \textit{asp1}^{D333A} strain under all conditions tested (Figure S2A-B, \cite{7}).

The \textit{A. nidulans} Asp1 orthologue AN5797.2 has the characteristic Vip1 family dual domain structure (Figure S5) and was named \textit{vlpA} \textit{Vip1}-like protein). To test if VlpA generates inositol pyrophosphates, biaxially expressed and purified GST-VlpA, which contains the putative kinase domain was used in the in vitro kinase assay \cite{54}. VlpA^{1-574} generated IP\(_7\) in an ATP dependent manner using IP\(_6\) as a substrate (Figure 6A, lanes 6, 4 and 5, respectively). This activity increased with increasing amounts of VlpA^{1-574} (Figure 6B).

We next deleted the endogenous \textit{vlpA} gene and found that the \textit{vlpA}\_deletion strain (\textit{\Delta vlpA}) showed a growth delay and smaller colonies than the wild-type strain (approximate 50\% diameter of colony on glucose medium) (Figure 6C). The majority of hyphae in the \textit{\Delta vlpA} strain displayed a normal morphology however swelling of hyphae was observed in some instances (Figure 6D, left). This phenotype could be caused by mis-positioning of the growth zone.

We constructed a strain expressing N-terminally GFP-tagged VlpA fusion protein under the control of the inducible \textit{alcA} promoter instead of native VlpA. Under repressed conditions with glucose as carbon source, the strain exhibited a growth delay (Figure 6C, bottom right most panel). Under de-repressed conditions with glyceral or induced conditions with threonine, the slow growth phenotype was alleviated implying that GFP-VlpA can complement the growth defect of the \textit{vlpA} deletion. We constructed a strain expressing GFP-VlpA under the native promoter and found that GFP-VlpA fluorescence was observed predominantly in the cytoplasm (Figure 6E, left).

Interestingly, the \textit{A. nidulans} VlpA is needed for correct growth zone selection as it is required for the correct positioning of the second germute. Once the first hypha reaches a determinate length, a second germ tube appears on the spore after the first septum at the base of the first hypha was formed \cite{70}. This second germination site normally lies opposite of the first hypha (Figure 6F). In \textit{A. nidulans}, MTs are formed from spindle pole bodies (SPB) and from septum-associated MT-organizing centers (septal-MTOCs) \cite{71,72}. MTs emanating from the septum of the first hypha grow towards the first germtube as well as into the septum and into the conidiophore metulae. The mutants also show irregular distribution of nuclei in vegetative hyphae \cite{73}. ApsB is a MTOC component that interacts with gamma-tubulin \cite{74}. The \textit{apsB} mutant shows an altered MT organization as it forms fewer MTs out of SPBs, compared to the wild-type and substantially fewer MTs from septa \cite{72}. We therefore analyzed such parameters in the \textit{vlpA}-deletion strain.

GFP tagged KipA, which is a kinesin localizing at growing MT plus-end, was used as plus-end marker to determine MT parameters \cite{71}. Comparing wild-type to the \textit{vlpA}-deletion strain during a five minute time period, we observed a reduction of newly emanating GFP-KipA signals in the \textit{vlpA}-deletion strain at SPBs (27\%) and at septal-MTOC (33\%) (Figure 6G, Figure S6, Movie S1 and S2). The growth rate of the MT plus-ends was slightly reduced in the \textit{vlpA}-deletion strain (21\%) (Figure 6H).

Pausing of MT plus-ends at hyphal tips was analyzed by using GFP-z-tubulin. Since the pausing time at hyphal tips was too short to determine if differences existed between the wild-type and the \textit{vlpA} deletion strains, we scored the number of MT plus-ends reaching hyphal tips during a 1 minute time period. We counted fewer MT plus-ends in the \textit{vlpA} deletion strain compared to the wild-type strain indicating that MT dynamics at the hyphal tip was altered in the absence of VlpA (Figure 6I).

The Asp1\_like protein UmAsp1 is important for proliferation and polar growth in \textit{U. maydis}

Finally, we studied the function of an Asp1 homologue in a distantly related fungus, the basidiomycte \textit{U. maydis}. Sequence comparison revealed a protein designated UmAsp1\textit{um06407} in MUMDB; MIPS \textit{Ustilago maydis} database \cite{75}, with 922 amino acids and 49\% sequence identity to \textit{S. pombe} Asp1 over its entire length (Figure S5). To study its function we generated deletion strains in laboratory strain AB33. This strain is a derivative of wild-type strain FB2 that contains an active hW2/hEl heterodimeric transcription factor under control of the nitrate-inducible promoter \textit{P}_{natr}. Thereby, b\_dependent filamentation can be elicited by changing the nitrogen source in the medium \cite{76}. We observed that a corresponding deletion strain of \textit{Umasp1} showed elevated proliferation during yeast-like growth in comparison to wild-type (Figure S7). Assaying TBZ sensitivity revealed that \textit{Umasp1} strains were hypersensitive to this MT inhibitor (Figure 7A; Figure S7B-C). For microscopic analysis we compared wild-type and \textit{Umasp1} strains expressing GFP-Tub1 (GFP fused to z-tubulin). The \textit{Umasp1} strain showed an increased number of cells that were clearly different from the cigar-shaped wild-type cells. Cells exhibited an increased diameter in the central region and/or were rounded-up at the poles (Figure 7B; Figure S8). Such cells were classified as having a disturbed shape and quantification revealed that about 40\% of \textit{Umasp1} cells had an abnormal cell morphology (Figure 7B).
Analysis of the MT cytoskeleton showed specific deviations from wild-type MTs. In wild-type cells 4 to 5 microtubule bundles are observed that are facing with their plus ends towards the poles [23,25,77]. We observed that the MT organization was altered in Umasp1Δ cells: a conservative quantification scoring only cells with drastic changes revealed that in comparison to the wild-type MT organization was altered (Figure S9). The most profound differences observed were (i) Umasp1Δ cells with large buds exhibited depolymerized MTs (Figure S9B, bottom panels); a phenotype rarely observed for wild-type cells. (ii) Umasp1Δ cells mostly with no bud or a small bud (early G2 phase) [23] had significantly more MT bundles. Instead of the 4 to 5 bundles present in wild-type cells, we observed 6 to 8 (Figure 7C–D; Movies S3 and S4). The fluorescence intensity of the GFP-Tub1 signal was drastically reduced in these bundles (Figure 7C, E) suggesting that loss of UmAsp1 results in an increased number of MT bundles with fewer MTs within such a bundle.

Studying the subset of intact MTs indicated that MT growth rate, which was analyzed by determining the velocity of the GFP-tagged U. maydis EBI protein Pep1 [77], was not significantly different compared to wild-type (Figure 7F), but the residence time of MTs pausing at the cell end was significantly reduced (Figure 7G). In summary, UmAsp1 is needed for correct morphology and MT organization during proliferation of yeast-like cells.

To investigate the function of UmAsp1 during hyphal growth, AB33 filamentation was induced on plates and in liquid medium. Wild-type forms a fuzzy colony indicative for efficient hyphal growth (Figure 8A, top, left panel). This was disturbed in Umasp1Δ strains (Figure 8A, top, right panel). Filaments were shorter, often bipolar and the amount of abnormal filamentous growth was clearly increased in Umasp1Δ strains (Figure 8B–C, Figure S10). Thus, as in hyphae of A. nidulans, UmAsp1 is important for filamentous growth.

To study the subcellular localization we generated strains expressing UmAsp1-GFP (C-terminal fusion to GFP). The resulting strain was phenotypically indistinguishable from wild-type (Figures 7A, 8A–C) demonstrating that the fusion protein is fully functional. Studying the subcellular localization in yeast or hyphal cells did not reveal any pronounced subcellular accumulation of the protein as has been shown for other Vip1-like proteins (Figure 8D). However, UmAsp1-GFP fluorescence was reduced in hyphae, suggesting that the protein amount decreases after filament induction (Figure 8E, Figure S11). Indeed, western blot analysis demonstrated that UmAsp1-GFP protein amounts decreased over time (Figure 8F). Thus, UmAsp1 protein amounts decrease and hence presumably intracellular inositol pyrophosphate levels appear to be down-regulated during the switch to hyphal growth.

Discussion

In this work we have defined the function of the C-terminal domain of the Vip1 family member Asp1 from S. pombe and have identified a new role for inositol pyrophosphates in fungal polarized growth and the modulation of MTs. In all three fungal model systems analyzed transport-based processes along the MT cytoskeleton are essential for proper polarized growth. However the long hyphal compartments of the filamentous fungi require a more sophisticated system of localized delivery [3,18,24]. Thus although Vip1-like proteins play a role in polarized growth in S. pombe, A. nidulans and U. maydis their specific roles are not expected to be identical.

Function of the Asp1 C-terminal histidine acid phosphatase domain

All Vip1 family members have a dual domain structure consisting of an N-terminal kinase domain and a C-terminal histidine acid phosphatase-like domain. Generation of inositol pyrophosphates has been shown for the budding yeast and human Vip1 family members [43–45,48]. In this work we have extended the analysis to two further fungal Vip1-like proteins: the S. pombe Asp1 and the A. nidulans VlpA. Both proteins generated inositol pyrophosphates in vitro. The use of Asp1 and VlpA N-terminal-only-variants mapped the kinase activity to the N-terminal part of the respective protein.

The precise function of the C-terminal phosphatase-like domain of Vip1-like proteins has been elusive. The histidine acid phosphatase signature motif is in principle present in Vip1-like proteins but the conserved “HD” motif has been replaced by H(I,V,A) [45]. A recent publication has shown that the phosphatase-like domains of the human Vip1 members are catalytically inactive. Instead the authors show that this domain plays a role in inositol lipid binding [48]. On the other hand, a comparison of the amounts of inositol pyrophosphates generated by human and the S. cerevisiae full-length Vip1 proteins versus N-terminal kinase-domain-only-variants, showed that the latter variants exhibited more specific activity [43,45]. This implied a negative impact of the phosphatase-like domain on inositol pyrophosphate production. However it was unclear, if this effect was due to the large size differences between the full length and the kinase-domain-only-variants [45]. In this paper we demonstrate that the phosphatase-like domain has a regulatory function: (i) the Asp1H397A variant generated significantly more inositol pyrophosphates in vitro than the equally sized wild-type Asp1 protein (Figure 9A). The Km values for these two proteins were similar, but V max for the mutant Asp1 variant Asp1H397A was higher. (ii) Addition of the phosphatase-only variant Asp1365-920 to an Asp1 protein contain-
Figure 7. Loss of Umasp1 causes aberrant morphology of and altered MT organization in U. maydis yeast cells. (A) Diagrammatic representation of growth inhibition test. The radius of growth zone inhibition was determined for the indicated strains on plates with 10 μl TBZ (concentration: 10 mg/ml) at the centre (experiments, n = 3. Error bars show SEM. ***, p < 0.001; t-test). Representative examples are shown on the right and in Figure S7C (size bar, 1 cm). (B) Percentage of cells with disturbed cell shape. Bars show the mean of three independent experiments with n > 100 cells (error bar shows SEM. *p < 0.03, t-test). Representative examples are shown on the right (size bar, 5 μm). (C) Top: Deconvolved fluorescence photomicrographs depicting MT morphology (via expression of GFP-Tub1 (GFP fused to α-tubulin)) of wild-type and Umasp1Δ cells (size bar, 5 μm). Note that due to deconvolution fluorescence for the Umasp1Δ cell appears brighter. Bottom: Corresponding intensity profile showing longitudinal maximum intensity of background subtracted raw images. (D and E) Whisker diagrams showing the number of wild-type and Umasp1Δ MT bundles (D) and their relative intensity (E). Whiskers indicate 90%/10% percentiles (n = 49 cells in (D) and n = 10 cells in (E); ***p < 0.001 Mann-Whitney test for (D) and (E)). (F) MT growth parameters. Growth of MTs was determined by analyzing the comet-like movement of Pep1-GFP (error
ing in vitro kinase assay massively reduced the IP$_7$ output (Figure 9A). However the presence of a mutated phosphatase variant, Asp1$_{965-970}$ H$_{973}$A in the assay did not have this effect (Figure S18). Thus our results suggest that the C-terminal phosphatase-like domain of Asp1 has enzymatic activity and its substrates are the inositol pyrophosphates produced by the N-terminal kinase domain of the protein (Figure 9B, model II). However as we have not formally proven that the C-terminal bars indicate standard deviation). Only MTs that grew >2 µm were analyzed (n = 225 and 125 for wild-type and Umasp1Δ, respectively). (G) The residence time of dynamic MTs was determined in GFP-Tub1 strains. n = 79 and 96 for wild-type and Umasp1Δ respectively. Error bar indicates standard deviation (unpaired t-test, *** p<0.001).

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Figure 8. Loss of UmAsp1 causes defects in filamentous growth. (A) Edges of colonies of the indicated AB33 derived strains grown on charcoal plates. Aerial hyphae are emanating from the colony. (B) Photomicrographs (DIC) of the indicated strains grown for 8 hrs under filament inducing conditions. Wild-type and Umasp1-GFP filaments form characteristic empty sections at the basal pole. White arrow: growth zone; white star: yeast cell (bar, 10 µm). (C) Bar diagram showing percentage of filaments exhibiting normal or disturbed growth. Bars show the mean of three independent experiments with n = 100 cells (error bar, SEM; ** p<0.0108). (D) Examples of Umasp1-GFP yeast cells and filaments (8 hours post induction) (bar, 10 µm). (E) Bar chart showing mean average fluorescence intensity of UmAsp1-GFP in yeast and hyphae (yeast, n = 10 cells and hyphae, n = 7 cells; see Figure S11 for example photomicrographs). Error bars indicate standard deviation (*** p<0.001 unpaired t-test). (F) Western blot analysis of protein extracts of strain AB33 Umasp1-GFP after induction (0–8 hrs) of filamentous growth. Tub1 served as a loading control (hpi, hours post induction).

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domain has phosphatase activity other modes of regulation are possible as shown in model I (Figure 9B).

We have shown previously that specific extrinsic signals appear to up-regulate Asp1 kinase activity via the cAMP PKA pathway [7]. We speculate that such an up-regulation might occur by modification and result in down-regulation of the Asp1 C-terminal domain function. Such a scenario could also be envisaged for other external signal induced processes regulated by Vip1 family members, such as the antiviral response [49].

Inositol pyrophosphate signaling is an important modulator of fungal growth

The present work has defined a new role for inositol pyrophosphates generated by the Vip1 family: the modulation of fungal growth and the MT cytoskeleton. In *S. pombe*, interphase MT organization and MT dynamics were strongly altered in the *asp1* mutant strains. In *A. nidulans* MT arrays from the SPB and the septal MTOC were affected in the *vlpA* deletion strain while in *U. maydis* loss of the Vip1-like protein resulted in increased TBZ sensitivity and an increase of cells with aberrant MT organization.

How then do inositol pyrophosphates modulate the MT cytoskeleton? In all systems tested to date and shown for *U. maydis* and *A. nidulans* in this work, Vip1 proteins are predominantly cytoplasmic without a specific subcellular localization [42,48]. However as inositol pyrophosphates appear to modulate processes by binding to proteins or by pyrophosphorylation of proteins, direct association of Vip1 proteins with their targets might not be necessary. Our analysis in *S. pombe* demonstrated that in the absence of the +TIPs EB1 family member Mal3 the MT cytoskeleton can still be modulated by inositol pyrophosphates. Furthermore MT localization of EB1 proteins appeared unaffected in the *S. pombe asp1* strain and the *U. maydis* strain. As the EB1 protein family is at the center of the +TIP network of MT plus-ends and required for the recruitment of the majority of +TIPs [17,59], we reason that such MT proteins are unlikely targets of inositol pyrophosphates. We have started to search for MT relevant inositol pyrophosphate targets by expressing either *asp1* (kinase domain only) or *asp1* (phosphatase domain only) in wild-type *S. pombe* mutants with an altered MT cytoskeleton. Our rationale is that the mutant phenotype of a strain with a deletion of a direct Vip1 target should not be affected by varying inositol pyrophosphate levels.

We found that inositol pyrophosphates show a "genetic interaction" with the MT plus-end components that can associate with MTs independently of EB1 (our unpublished observations). However other MT structures might also be modulated by inositol pyrophosphates: MTs emanating from SPBs and septal MTOCs are reduced in the *A. nidulans vlpA*-deletion strain as has been shown for the *apsB* mutant strain [72]. ApsB is a conserved MTOC associated protein that interacts with γ-tubulin [74].

Of particular interest is the observed direct correlation between intracellular inositol pyrophosphate levels and the time that *S. pombe* MT plus-ends stay at the cell tip before a catastrophe event. Components of a fungal growth zone can regulate MT plus-end dynamics as has been shown for *A. nidulans* Tea1 family member TeaA, which negatively regulates the activity of the XMAP215 protein AlpA [19]. Thus it is feasible that Asp1 enzymatic activity regulates MT dynamics at the cell tip. Although immunofluorescence analysis of *S. pombe* Asp1-GFP did not show a specific cytoplasmic localization [42], localization of the human Vip1 member PPiP5K1 was slightly enhanced at the plasma membrane [48]. Plasma membrane targeting of PPiP5K1 in NIH3T3 cells was increased dramatically following PtdIns3 kinase activation [48].

Inositol pyrophosphates and growth zone selection

In fission yeast the switch from mono- to bipolar growth (NETO) is a complicated process that is regulated by a number of interwoven processes [10,12]. These range from the correct positioning of landmark proteins by the MT cytoskeleton to the
Inositol Pyrophosphates Regulate Microtubules

Materials and Methods

In vitro enzymatic activity of Vip1-like proteins

PCR-generated DNA fragments containing the S. pombe asp1\(^{11937A}\), asp1\(^{12334}\), asp1\(^{H397A}\) or asp1\(^{1264-920}\) ORFs, the S. cerevisiae VIP1\(^{5-533}\) and A. nidulans VlpA\(^{1-574}\) (kinase domain) were cloned into E. coli expression vector pKM36 (a gift from Dr. K. Møllekæn, Heinrich-Heine-Universität, Düsseldorf, Germany) to generate GST-tagged proteins. These proteins were expressed and purified from E. coli strain Rosetta (DE8) according to protocol (Sigma Aldrich). Protein concentration was determined using Bradford. Defined quantities of the Vip1-like proteins were used in an enzymatic reaction followed by PAGE analysis [54]. Intensity of IP\(_7\) bands was determined with Image J 1.44 (NIH). Determination of \(K_m\) and \(V_{max}\). Enzymatic reactions with 2 \(\mu\)g of protein were carried out for 6 hours using 0–300 \(\mu\)M IP\(_7\) substrate. The amount of IP\(_7\) generated per reaction was determined by quantifying the relevant IP\(_7\) band and converting this number using an IP\(_7\) calibration curve. IP\(_7\) was obtained from Sigma-Aldrich. Michaelis-Menten enzyme kinetics were calculated with GraphPad Prism6 (GraphPad Software, Inc.).

Strains and media

All strains used are listed in Table 3. S. pombe strains were grown and new strains were obtained as described [7]. A. nidulans was grown in supplemented minimal medium including 2% glucose, 2% glycerol or 2% threonine [80]. A. nidulans strain constructions were as described [81]. To generate a N-terminal GFP fusion construct of VlpA a 900 bp fragment of vlpA (starting from ATG) was amplified from genomic A. nidulans DNA with appropriate primers. This AseI-PacI-digested PCR fragment was cloned into the corresponding sites of pCMB17apx for N-terminal GFP fusion proteins of interest expressed under the control of alcA promoter, containing Neurospora crassa pyr4 as a selective marker [82], generating pCoS105. The 1.5-kb promoter of vlpA was amplified from genomic DNA with appropriate primers and cloned into the corresponding sites of pCoS105, generating pCoS228. They were transformed into wild-type strain TN02A3. To express Vip1 variant GFP-VlpA\(^{1-574}\) (contains the kinase domain) from the alcA promoter, the fragment of vlpA was amplified from genomic A. nidulans DNA with appropriate primers. This AseI-PacI-digested PCR fragment was cloned into the corresponding sites of pCMB17-pyroA (pyr-4 was replaced with pyrA in pCMB17apx), generating pCoS197, which was transformed into the wild-type strain TN02A3 and vlpA-deletion strain. Integration event was confirmed by PCR. vlpA was deleted via transformation of a deletion cassette (Program Project grant GM068087) into TN02A3 and the deletion confirmed by southern blotting. U. maydis strain constructions and growth of yeast like cells was performed according to published protocols [76]. Filamentous growth of AB33 and variants was induced by shifting 20 or 50 ml of exponentially growing cells (OD600 = 0.4–0.5) from complete medium (CM) to nitrate minimal medium each supplemented with 1% glucose. Cells were incubated at 28°C shaking at 200 rpm for 4 to 8 h prior to microscopy. For serial dilution patch tests, cells were pre-grown to OD600 = 0.5 before plating. For quantitative inhibition studies, cells were grown to OD600 = 0.5 and 300 \(\mu\)l were streaked out on a CM-plate. The filter paper present at the plate centre contained either 10 \(\mu\)l DMSO (solvent control) or 10 \(\mu\)l TBZ (10 mg/ml). After three days of growth at 28°C the radius of growth inhibition was measured.

Generation of asp1 variant containing plasmids and western blot analysis

asp1\(^{H367F}\), asp1\(^{1-364}\) (plasmid p672), asp1\(^{1937A}\) plasmids are derivatives of pJR2-3XL and have been described previously [7]. For the asp1\(^{1-367-asp1H397A}\) containing plasmid, p672 was cut with Asp1 and a PCR generated DNA fragment containing the nmt1\(^{36}\) promoter followed by the DNA sequence encoding asp1\(^{1-365-520}\) inserted via homologous recombination in S. cerevisiae [83]. asp1\(^{R397A}\) and asp1\(^{H607}\) were generated by directed mutagenesis using the QuikChangeII Site-Directed Mutagenesis Kit (Stragene) and after verification of sequence by sequence analysis cloned into pJR-3XL via S. cerevisiae asp1H397A containing DNA sequences were fused to gfp and expression of the fusion protein was determined by western blot analysis as has been described [7]. U. maydis Vip1G expression was determined via western blot analysis as has been described [36].

Microscopy

For imaging of living S. pombe cells, cells were pre-grown in minimal medium at 25°C or 30°C and slides were prepared by mounting cells on agarose pads as described in [84]. Images were obtained at room temperature using a Zeiss Spinning Disc confocal microscope, equipped with a Yokogawa CSU-X1 unit and a MRm Camera. Slides were imaged using AxioVision.
Table 3. Strains used in this study.

### S. pombe

| name      | genotype                        | source         |
|-----------|----------------------------------|----------------|
| UFY1156   | h^{+} asp1::kan^{R} his3-D1 ade6-M216 leu1-32 ura4-D18 | U. Fleig       |
| UFY605    | h^{+} his3-D1 ade6-M210 leu1-32 ura4-D18               | K. Gould       |
| UFY1579   | h^{+} asp1^{H397A::kan^{R}} his3-D1 ade6-M210 leu1-32 ura4-D18 | U. Fleig       |
| UFY1511   | h^{+} asp1^{H397A::kan^{R}} his3-D1 ade6-M210 leu1-32 ura4-D18 | U. Fleig       |
| UFY857    | h^{+} kan::nm781::gfp::atb2 leu1-32                     | T. Toda        |
| UFY963    | h^{+} kan::nm871::gfp::atb2 ade6-M216 his3D1 leu1-32   | This study     |
| UFY1318   | h^{+} kan::nm871::gfp::atb2 ade6-M210 his3D1 leu1-32 ura4-D18 | This study     |
| UFY1763   | h^{+} asp1^{H397A::kan^{R}} his3-D1 ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY1529   | h^{+} asp1^{H397A::kan^{R}} his3-D1 ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY1407   | h^{+} asp1::kan^{R} his3-D1 leu1-32 ade6-M216           | This study     |
| UFY135    | h^{+} malA::his3^{Δ} his3Δ ade6-M210 leu1-32 ura4-D18   | U. Fleig       |
| UFY1561   | h^{+} malA::ura4^{+} ade6-M210 his3Δ leu1-32 ura4-D18   | U. Fleig       |
| UFY1641   | h^{+} asp1^{H397A::kan^{R}} malA::his3^{Δ} his3Δ ade6-M210 leu1-32 ura4-D18 | U. Fleig       |
| UFY1528   | h^{+} asp1^{H397A::kan^{R}} malA::his3^{Δ} his3Δ ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY1322   | h^{+} asp1::kan^{R} malA::his3^{Δ} his3Δ ade6-M216 leu1-32 ura4-D18 | This study     |
| UFY1729   | h^{+} asp1::kan^{R} malA::his3^{Δ} ade6-M210 leu1-32 ura4-D18 | U. Fleig       |
| UFY2164   | h^{+} asp1::kan^{R} malA::his3^{Δ} ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY880    | h^{+} asp1::kan^{R} malA::ura4^{+} his3Δ ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY1762   | h^{+} asp1^{H397A::kan^{R}} malA::ura4^{+} his3Δ ade6-M210 leu1-32 ura4-D18 | This study     |
| UFY596    | h^{+} malA::pk-GFP::ura4^{+} ade6-M210 his3D1 leu1-32 ura4-D18 | H. Browning    |
| UFY2015   | h^{+} asp1^{H397A::kan^{R}} malA::ura4^{+} ade6-M210 his3D1 leu1-32 ura4-D18 | This study     |
| UFY2014   | h^{+} asp1^{H397A::kan^{R}} malA::ura4^{+} ade6-M210 his3D1 leu1-32 ura4-D18 | This study     |
| UFY1582   | h^{+} asp1^{H397A::kan^{R}} ade6-M210 leu1-32 ura4-D18 his3-D1 | This study     |

### A. nidulans

| name      | genotype                        | source         |
|-----------|----------------------------------|----------------|
| TN02A3    | pyrG89, argB2, ΔnkuA::argB, pyroA4 | [88]           |
| SDV25     | alcA::gfp::kipA::pyr-4, pyrG89, aipA6 | [72]           |
| SSK92     | alcA::gfp::kipA::pyr-4, pyrG89, pyroA4 | [71]           |
| SCO590    | alcA::gfp::vlpA::pyr-4, pyrG89, argB2, ΔnkuA::argB, pyroA4 | This study     |
| SCO576    | vlpA::gfp::vlpA::pyr-4, pyrG89, argB2, ΔnkuA::argB, pyroA4 | This study     |
| SCO5153   | alcA::gfp::vlpA::pyr-4, pyrG89, argB2, ΔnkuA::argB, pyroA4 | This study     |
| SCO5155   | alcA::gfp::vlpA::pyr-4, pyrG89, argB2, ΔnkuA::argB, pyroA4 | This study     |
| SCO594    | ΔvlpA::pyr-4, pyrG89, argB2, ΔnkuA::argB, pyroA4 | This study     |
| SDO2      | ΔvlpA::pyr-4, alcA::gfp::kipA::pyr-4 | This study     |

### U. maydis

| name      | genotype                        | source         |
|-----------|----------------------------------|----------------|
| AB33      | a2 P_{asp}::bW2bE1               | [76]           |
| UM299     | a2 P_{asp}::bW2bE1 P_{mnr::rmm4R::NatR} P_{meI::ub1Gn_CbxR} | P. Becht       |
| UM871     | a2 P_{asp}::bW2bE1 P_{meI::ub1Gn_CbxR} | S. Baumann     |
| UM830     | a2 P_{asp}::bW2bE1 P_{mnr::rmm4C_HyyR} | [35]           |
| UM958     | a2 P_{asp}::bW2bE1 P_{asc1::HygR} | This study     |
| UM960     | a2 P_{asp}::bW2bE1 P_{asp1::G2::HygR} | This study     |
| UM1112    | a2 P_{asp}::bW2bE1 P_{mnr::rmm4R::NatR} P_{meI::ub1Gn_CbxR} P_{asc1::HygR} | This study     |
| UM1158    | a2 P_{asp}::bW2bE1 P_{meI::ub1Gn_CbxR} P_{asc1::HygR} | This study     |

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software. Images shown are maximum intensity projections of 10–25 z-slices of 0.24–0.5 μm distance. For measurement of MT dynamics, strains expressing GFP-Atb2 [57] under control of the nmt81 promoter were pre-grown under promoter-derepressing conditions for at least 48 hrs. For technical reasons, we used the nmt81::gfp-atb2 construct, as this facilitated the measurement of the sometimes faint MTs of the asp1D333A strain. Time-lapse images were acquired in 5–10 sec intervals.

For live-cell imaging of *A. nidulans* germlings and young hyphae, cells were grown on coverslips in 0.5 ml of Supplemented minimal media with 2% glycerol (de-repression of the *oclA* promoter, moderate induction). Cells were incubated at 30°C overnight/1 day. Coverslips were mounted on slide glass. Tempcontrol mini (Pepcom) was used for a constant temperature of the slide glass during microscopy. Images were captured using an Axiohot microscope using a Planapochromatic 63 times oil immersion objective lens, the Zeiss AxioCam MRM camera and the HBO103 mercury arc lamp (Osrarm) or HXP 120 (Zeiss, Jena, Germany). Images were collected and analyzed with the AxioVision software. ImageJ software.

Live cell imaging of *U. maydis* was performed according to published protocols [36]. Microscope and camera were controlled by MetaMorph (Version 7.7.0.0, Molecular Devices, Seattle, IL, USA). The same software was used for measurements and image processing including the adjustment of brightness and contrast. MT bundles were visualized with a 63× Planapochromatic (NA 1.4, Zeiss) or 100× α-Planapochromatic (NA 1.46, Zeiss) in combination with a HXP lamp or laser illumination (488 nm), respectively. Z-stacks were composed of 38 planes with 270 nm spacing (63×) and 66 planes with 240 nm spacing (100×). Exposure time was 100 ms. Deconvolution was performed with Fiji. A theoretical PSF was determined with the differfract PSF 3D plugin and images were generated using the Deconvolve 3D plugin [83,86]. 3D movies were generated with MetaMorph. To determine the number of MT bundles z-stacks were collapsed to a maximum projection and after cytoplasmic background subtraction the number of bundles was determined. For determination of MT bundle intensity the maximum values of a longitudinal slice scan (Fig. 7C) were plotted over distance. Each value from the x-axes was included in a whisker diagram (Fig. 7E) showing the median and range of fluorescent MT bundles (*n* = 10 cells for wild-type and Unasp1Δ, respectively). Fluorescence micrographs of Unasp1-GFP were acquired with 500 ms exposure time in a single plane. Before determining average cytoplasmic fluorescence images were background subtracted. For measurement of MT growth (Fig. 7F) strains expressing GFP-Tub1 were used. Z-stacks were composed three planes with 1 μm spacing (100× objective). Exposure time was 100 ms. For measurement of MT residence time (Fig. 7G) strains expressing Pebl-GFP were used. Z-stacks were composed of 5 planes with 800 nm spacing (100× objective). Exposure time was 100 ms. Statistical analysis was done with Prism5 (Graphpad).

**Supporting Information**

**Figure S1** Asp1 converts IP₆ to IP₇ in vitro. (A) Left panel: S. cerevisiae Vip1 for which enzymatic activity had been demonstrated was used as a positive control for IP₇ generation [43]. 1 μg bacterially expressed and purified GST-Vip1D333A (contains kinase domain) was used in an enzymatic reaction as described [54] followed by resolution of the products via PAGE and staining of the gel with Toluidine Blue. −, component not present in assay; +, component present in assay. Right panel: Asp1 generates IP₇ from IP₆ in an ATP-dependent reaction. 1 μg bacterially expressed and purified GST-Asp1 was used in the above mentioned in vitro assay. (B) Correlation between Asp1 protein input and the amount of IP₇ generated. Left panel: Toluidine Blue stained PAGE showing IP₇ produced by varying amounts of GST-Asp1 protein. Incubation time: 16 hrs. Right panel: Diagrammatic representation of the quantification of the IP₇ bands shown in the left panel. (TIF)

**Figure S2** (A) asp1D333A, asp1Δ and asp1D333A, H397A strains show TBZ hypersensitivity. Serial dilution patch tests (10⁻⁵–10⁻¹ cells) of the indicated strains on YESS plates with (+) or (−) without TBZ. Plates were incubated for 5 days at 25°C. (B) asp1D333A and asp1Δ strains are sensitive to NaCl and caspofungin and resistant to treatment by the cell wall enzyme zymolyase. Serial dilution patch tests (10⁻⁵–10⁻¹ cells) on YESS plates with (+) or (−) without (−) 50 mM NaCl or 1.5 μg/ml caspofungin, respectively. Plates were incubated for 4 days at 25°C. For zymolyase experiments cells were incubated with zymolyase and OD₆₀₀ determined at the indicated time intervals. Reduction in OD₆₀₀ is due to cell lysis. (TIF)

**Figure S3** Expression of plasmid-borne asp1Δ variants in the asp1Δ strain. (A) Diagrammatic representation of the *S. pombe* LEU2 plasmids used in B–C. P, nmt1 promoter. (B) Western blot analysis of the asp1Δ strain expressing the indicated Asp1-GFP variants. Similar amounts of protein were resolved by SDS-PAGE and probed with an anti-GFP antibody or an anti-α-tubulin antibody (loading control). (C) Quantification and diagrammatic representation of the Asp1-GFP signals obtained in (B). (TIF)

**Figure S4** (A) Serial dilution patch tests (10⁻⁵–10⁻¹ cells) of the indicated strains grown on minimal medium without thiamine (promoter on conditions) for 5 or 4 days at 25°C or 30°C, respectively. Incubation on TBZ containing plates was for 9 days at 25°C. (B) Live cell images of the indicated strains expressing gfp-tub2Δ. Time between the images is 10 seconds. In each case the arrow indicates a short MT that polymerizes from the cell middle but is not oriented along the long axis of the cell. In the wild-type strain this MT reaches the cell cortex (80 seconds image), becomes deflected and continues to grow. In the asp1D333A strain, such a MT touches the cell cortex (100 second image) and then depolymerizes. Bars, 5 μm. (TIF)

**Figure S5** (A) Sequence comparison of the Vip1 family members from *S. pombe*, *S. cerevisiae*, *A. nidulans* (AN5797.2) and *U. maydis* (UM06407.1). Multiple sequence alignment was performed with MultAlin using BLOSUM62 matrix [87]. (B) The respective kinase and phosphatase domains are indicated in green and grey, respectively. (TIF)

**Figure S6** GFP-KipA, a marker of growing MT plus-ends, in the wild-type (SSK92) and the vlpA-deletion strain (SDO2). (A) Diagrammatic representation of the components shown in (B) and (C). (B) and (C) We compared newly emanating GFP-KipA signals in the wild-type (B) and the *vlpA* strain (C) during a 5 minute time period at SPBs (asterisks) and at septal-MTOC (white arrows). Bar, 10 μm. Kymographs at septa during a 5 minute time period are shown. GFP signals coming from the septum are shown by blue arrows. GFP signals arriving at the septum are shown by red arrows. Bar, 1 μm. (TIF)
Figure S7 Loss of UmAsp1 causes defects in proliferation and leads to TBZ sensitivity. (A) Growth of the indicated yeast strains over time. (B) Serial dilution patch test (10⁷ to 10² cells) of the indicated strains grown with/without 10 μg/ml TBZ. (C) Filter paper with/without 10 μg/ml TBZ was placed on a lawn of U. maydis cells (strains indicated above). The region indicated by a white bar was measured to determine the zone of inhibition (radius in cm) given in Figure 7A. Note, plates of Umash1Δ cells appear slightly darker due to secretion of an unknown pigment.

Figure S8 Loss of UmAsp1 causes alterations in cell morphology. Representative DIC images of wild-type (A) and Umash1Δ (B) cells, quantified in Figure 7B are shown (Bars, 10 μm).

Figure S9 Loss of UmAsp1 causes defects in MT organization. Representative fluorescence images of wild-type (A) and Umash1Δ (B) cells are shown. (C) The indicated MT categories were determined in wild-type and Umash1Δ strains. Bars show the mean of three independent experiments with n>100 cells (error bars show SEM, p<0.0001; two-way ANOVA test).

Figure S10 Loss of UmAsp1 causes defects in filamentous growth. Representative DIC images of wild-type (A) and Umash1Δ (B) hyphae 8 hours after filament inducing conditions (Bars, 10 μm). Quantification is shown in Figure 8C.

Figure S11 UmAsp1-GFP signal decreases during switch to filamentous growth. Fluorescence micrographs of mixed cultures expressing either UmAsp1-GFP (*) or Rrm4-mCherry (#) [35] are shown. Micrographs detecting either green or red fluorescence (seen in the Rrm4-mCherry control) can be judged.

Figure S12 (A) Time dependent generation of IP₇ by GST-Asp1 variants. 4 μg of the indicated proteins were used in an ATP-dependent enzymatic reaction and the resulting inositol pyrophosphates were resolved on a 35,5% PAGE and stained with Toluidine Blue. −, component not present; +, component present. (B) Quantification and diagrammatic representation of the IP₇ bands obtained in the assay shown in (A).

Figure S13 IP₆ amounts in the presence (+) or absence (−) of 9 μg Asp1365-920. Assay conditions and detection of IP₆ were as described for the in vitro kinase assay.

Figure S14 Western blot analysis of the asp1Δ strain expressing the indicated Asp1-GFP (arrow shows full length fusion protein) variants. Similar amounts of protein were resolved by SDS-PAGE and probed with an anti-GFP antibody or an anti-α-tubulin antibody (left and right panels, respectively).

Figure S15 Percentage of MTs polymerizing towards the lateral cortex (black bars) or towards a cell end (white bars). Wild-type: n = 77, asp1ΔH397A, n = 73, asp1ΔD333A, n = 63.

Figure S16 Diagrammatic representation of the number of interphase MTs in the indicated strains (mal3Δ strain, n = 95; mal3Δ asp1ΔH397A strain, n = 99).

Figure S17 Movement of outmost outbound Tea2-GFP comets (see diagram). Speed of comets (nm/sec); wild-type, 60±26.7, n = 64; asp1ΔD333A, 90±43.3, n = 71. *p<0.0005 for asp1ΔD333A vs. wild-type (Welch-test).

Figure S18 Generation of IP₇ by GST-Asp1 with varying amounts (2,4,8 μg) of Asp1363-920 H397A. Enzymatic reaction was carried out as described in Figure 1C. −, component not present; +, component present.

Movie S1 GFP-KipA, a marker of growing MT plus-ends in the wild-type strain (SSK92), 2 seconds intervals, total 5 minutes. Scale bar, 10 μm.

Movie S2 GFP-KipA in the vlpA-deletion strain (SDO2). 2 seconds intervals, total 5 minutes. Scale bar, 10 μm.

Movie S3 3D reconstruction of a wild-type cell expressing GFP-Tub1. The underlying z-stack is depicted in Figure 7. Size of angle images of the z-stack was doubled and pixels resampled. Ratio of xy-distance and xz-distance was chosen 1:1 to obtain cubic voxels. Movie comprises 14 frames in 12 seconds.

Movie S4 3D reconstruction as in Movie S3 of an Umash1Δ cell expressing GFP-Tub1.

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Conceived and designed the experiments: NT MF RF UF. Performed the experiments: JP CR CS TP VJ EW PR SB. Analyzed the data: JP CR CS TP VJ EW PR NT MF RF UF. Contributed to the writing of the manuscript: NT MF RF UF.

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