Asp1 bi-functional activity modulates spindle function via controlling cellular inositol pyrophosphate levels in *Schizosaccharomyces pombe*

running title: Vip1 pyrophosphatase

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Abstract

The generation of two daughter cells with the same genetic information requires error-free chromosome segregation during mitosis. Chromosome transmission fidelity is dependent on spindle structure/function which requires Asp1 in the fission yeast *Schizosaccharomyces pombe*. Asp1 belongs to the PPIP5Ks/Vip1 family which generates high energy inositol pyrophosphate (IPP) molecules. Here we show that Asp1 is a bi-functional enzyme *in vivo*: Asp1 kinase generates specific IPPs which are the substrates of the Asp1 pyrophosphatase. Intracellular levels of these IPPs directly correlate with microtubule stability: pyrophosphatase loss-of-function mutants raised Asp1-made IPP levels twofold thus increasing microtubule stability while overexpression of the pyrophosphatase decreased microtubule stability. Absence of Asp1-generated IPPs resulted in an aberrant increased spindle association of the *S. pombe* kinesin-5 family member Cut7 which led to spindle collapse. Thus, chromosome transmission is controlled via intracellular IPP levels. Intriguingly, identification of the mitochondria-associated Met10 protein as the first pyrophosphatase inhibitor revealed that IPPs also regulate mitochondrial distribution.
Introduction

Inositol pyrophosphates (IPPs) are signaling molecules present in all eukaryotes and are synthesized by the two enzyme families IP6Ks Kcs1 and PPIP5Ks/Vip1 (1-3). Numerous cellular processes are regulated by these high energy molecules including the activation of innate immune response in mammals and plants, insulin signaling, telomere length maintenance and cell death (4-8). IPP generating enzymes control cell morphogenesis in fungi including that of human fungal pathogens (9-11). In the fission yeast S. pombe the PPIP5Ks/Vip1 family member Asp1 is essential for the adaptation to nutrient limitation resulting in the dimorphic switch which allows yeast cells to grow in a substrate-invasive pseudohyphal manner (9). Alteration of the interphase microtubule (MT) cytoskeleton is an important contributor for efficient pseudohyphal growth in S. pombe (9) and Asp1 is needed for stability of interphase MTs (10). Assembly and function of the mitotic spindle also relies on Asp1: S. pombe cells expressing specific asp1 variants show aberrant bipolar spindle formation due to altered MT dynamics and spindle forces plus defects at the kinetochore-microtubule interface leading to chromosome missegregation (12).

Two mechanisms have been described for modulation of biological processes by IPPs: pyrophosphorylation or the reversible binding to a protein (13, 14). IPP protein targets appear to be numerous as more than 150 S. cerevisiae proteins were isolated in a screen using inositol polyphosphates/pyrophosphates as bait (15).

The best studied IPPs are the two diphosphoinositol pentakisphosphate isoforms, 1-IP$_7$ and 5-IP$_7$, and bis-diphosphoinositoltetrasphosphate 1,5-IP$_8$. They are synthesized from inositol hexakisphosphate (named IP$_6$ in the text) by two classes of enzyme families: IP6Ks/Kcs1 and PPIP5Ks/Vip1. Synthesis of 5-IP$_7$ is carried out by
the 5-kinase activity of IP6Ks/Kcs1 (16, 17) while PPPIP5Ks/Vip1 can add a diphosphate group to position 1 of IP₆ or 5-IP₇ thus generating 1-IP₇ and 1,5-IP₇ (named IP₆ in the text), respectively (1, 2, 18, 19). The physiological in vivo substrate(s) of the kinase domain of the PPPIP5Ks/Vip1 family has not been easy to define in a number of organisms analyzed to date (1-3). However, HPLC analysis of inositol phosphates in an *S. cerevisiae* VIP1 deletion strain suggested that Vip1 kinase activity might be responsible for the generation of IP₆ as has been demonstrated for the PPPIP5Ks/Vip1family members in *Cryptococcus neoformans* and *Arabidopsis thaliana* (6, 11, 20). Also in mammalian cells PPPIP5K are mainly responsible for IP₆ synthesis since only <2% of the IP₇ pool is synthesized by PPPIP5K (21).

Cellular IPP levels can be altered upon extrinsic signals. The jasmonate-mediated wound response of *A. thaliana* led to an increase of IP₆ (6). In *D. discoideum* IPPs are greatly increased during the chemotactic response (22) while in mammalian cells IP₆ levels are elevated upon hyperosmotic stress (3, 23). The mechanism(s) by which the relative abundance of IPPs is regulated is not understood. However, enzymes exist that can dephosphorylate IPPs in a non-specific (24-26) or specific manner(27). Thus down-regulation of such enzymes might contribute to increased cellular IPP pools.

In this context, the C-terminal domains of PPPIP5Ks/Vip1 family members are of particular interest. PPPIP5Ks/Vip1 proteins have a N-terminal kinase domain and a C-terminal domain with homology to histidine-acid-phosphatases (1). The site signature motif of histidine-acid-phosphatases RHxxR and HD (28) is present in PPPIP5Ks/Vip1 family members except for the aspartate next to the second histidine (1). Nevertheless, the C-terminal domain of the *S. pombe* PPPIP5Ks/Vip1 member Asp1
has pyrophosphatase activity \textit{in vitro} (10), which is inhibited by iron-sulfur clusters and is specific for the hydrolysis of the pyrophosphate at position 1 of the inositol ring (29).

In this work, we have dissected the function of the Asp1 kinase and pyrophosphatase domains \textit{in vivo} and found that they control intracellular IP$_8$ levels and thus the biological processes that require these specific IPPs.
Results

The Asp1 kinase domain is responsible for the generation of IP₈ in vivo

To analyze the in vivo function of the Asp1 kinase domain we measured inositol polyphosphates in a wild-type strain and the two mutant strains asp₁D₃₃₃ₐ and asp₁Δ. The amino acid D333 is a key catalytic residue required for Asp1 kinase activity (2), while the entire asp₁+ gene has been deleted in the asp₁Δ strain (9).

Inositol polyphosphates had not been assayed in S. pombe cells before and thus we first defined the growth conditions needed. S. pombe is a natural inositol auxotroph and requires inositol in the media ((30) and our observations). 10 µM inositol was the minimum concentration required for normal cell growth. Thus, cells of the three strains were radiolabeled with [³H]inositol in the presence of 10 µM cold inositol.

Next, soluble inositol polyphosphates were extracted, fractions separated by HPLC and quantified by scintillation counting (31). The wild-type strain showed three prominent peaks; the most abundant was IP₆, followed by IP₇ and IP₈ (Fig 1A, for standards profile see Fig S1). In the asp₁D₃₃₃ₐ and asp₁Δ strains, the IP₈ peak was absent and the IP₇ peak increased (Fig 1B and 1C, quantification in 1D). Thus Asp1 kinase has enzymatic function in vivo generating IP₈ via the IP₇ substrate. We have shown previously that strains without functional Asp1 kinase have defects in two biological processes: (i) chromosome segregation and (ii) the dimorphic switch (9, 10, 12). We can conclude now that these processes require IP₈.

Asp1 pyrophosphatase activity leads to destabilized MTs and an inability to switch to pseudohyphal invasive growth
To understand the in vivo function of the Asp1 C-terminal domain, we assayed the consequences of overexpression of wild-type and mutant variants on MT stability and the dimorphic switch. The mutant Asp1 C-terminal proteins were generated by mutating conserved amino acids of the histidine acid phosphatase signature motifs (Fig 2A, M1 and M2, respectively) (1).

A strain expressing the wild-type Asp1 C-terminal domain (amino acids 365-920, Fig 2A) from the thiamine-repressible nmt1+ promoter was hypersensitive to the MT poison thiabendazole (TBZ) demonstrating that expression of the wild-type pyrophosphatase domain decreased MT stability (Fig 2B, middle panels) (10). However expression of mutant $asp1^{365-920/H397A}$ (mutation at position H397 in M1 motif, Fig 2A) did not lead to TBZ hypersensitivity (Fig 2B, bottom panels) indicating that $asp1^{365-920/H397A}$ was non-functional. Protein expression levels of Asp1$^{365-920}$ and Asp1$^{365-920/H397A}$ were comparable (Fig S2).

Similarly, the ability to grow in an invasive pseudohyphal manner was abolished in cells expressing $asp1^{365-920}$. A wild-type strain expressing $asp1^{365-920}$ on a plasmid via the nmt1+ promoter could not grow invasively (Fig 2C, bottom middle panel). Growth per se was not affected in $asp1^{365-920}$ expressing cells (Fig 2C, surface growth). On the other hand, $asp1^{365-920/H397A}$ expressing cells grew invasively in numbers comparable to the control (Fig 2C, bottom right and left panel, respectively; quantification on the right).

To understand the possible effect of $asp1^{365-920}$ and $asp1^{365-920/H397A}$ expression on intracellular IPP levels we measured inositol polyphosphates in strains expressing these variants. As expected the wild-type strain transformed with the vector control showed the three peaks for IP$_6$, IP$_7$ and IP$_8$ (Fig 3A). Expression of $asp1^{365-920}$ massively decreased IP$_8$ levels and increased IP$_7$ in comparison to the control (Fig
3B and quantification in 3D). Thus, Asp1^{365-920} has in vivo pyrophosphatase activity and the substrate is IP₈.

The inositol polyphosphate profile of asp₁^{365-920/H397A} expressing cells did not decrease IP₈ levels as shown for wild-type pyrophosphatase expression (Fig 3B and C and quantification in 3D), demonstrating that this Asp1 variant was enzymatically inactive in vivo. In fact, the HPLC profile of asp₁^{365-920/H397A} expressing cells consistently showed higher IP₈ peaks than the control strain (Fig 3D). This result raises the interesting possibility that Asp1^{365-920/H397A} acts as a “dominant negative” that might titrate away a protein/protein complex required for activation of the wild-type pyrophosphatase.

In summary, the Asp1 C-terminal domain has enzymatic activity in vivo using IP₈ as substrate. IP₈ is required for MT stability and the ability to switch to pseudohyphal invasive growth. Thus the Asp1 pyrophosphatase domain negatively regulates these two biological processes (Fig 3E).

Correct spindle formation requires the concerted action of several motor proteins and we have shown previously that ectopic expression of the Asp1 pyrophosphatase domain rescued the temperature-sensitive lethal phenotype of a cut7^{-446} strain (12). Cut7 belongs to the kinesin-5 Eg5 family of motor proteins, which localize to the spindle midzone and the spindle poles supporting bipolar spindle assembly (32). We assayed the consequences of asp₁^{365-920} expression on mitotic Cut7 localization in a strain endogenously expressing cut7^{-GFP} (33). Live-cell imaging of short spindles (2-3.5 μm) of a cut7^{-GFP} strain transformed with a control plasmid, revealed fluorescence mainly at the two spindle poles and the spindle midzone (Fig 4A). Cells expressing asp₁^{365-920} had a significantly increased Cut7-GFP spindle midzone signal compared to control cells: quantification of the Cut7-GFP signal at the spindle middle
in relation to the spindle pole signals (Fig 4B) revealed that \(asp1^{365-920}\) expression led to an abnormal increase of Cut7-GFP fluorescence on the spindle (Fig 4C).

We had shown previously that \(asp1^{D333A}\) mitotic cells showed spindle breakage of short spindles prior to sister chromatid separation (12). Spindle collapse was also observed in \(cut7^-\)-GFP cells expressing plasmid-encoded \(asp1^{365-920}\) (Fig 4D). 30% of such analyzed cells showed short spindles (<4.5 μm) that collapsed between one to three times during our analysis (Fig 4D, Suppl. Movie S1). Interestingly, \(asp1^{365-920}\) expressing cells with breaking spindles showed significantly higher Cut7-GFP spindle midzone fluorescence than cells with non-breaking spindles (Fig 4E) suggesting that spindle collapse might be mediated by abnormally high amounts of Cut7 on the spindle.

Cut7-GFP signal intensity was also assayed in an \(asp1^{D333A}\) background. Again we found that in the absence of Asp1 generated IP8, Cut7-GFP spindle fluorescence was increased significantly (Fig 4F).

The human Eg5 kinesin-5 member is up-regulated in many types of cancer, a feature that correlates with poor prognosis (34). \(S.\ pombe\) cells without functional Asp1 kinase have defects in bipolar spindle formation and increased chromosome missegregation (12). As aberrant expression of human Eg5 results in polyploid cells in a mouse system (35) we re-examined chromosome segregation in IP8-less \(asp1^{D333A}\) yeast strains. Time lapse images of \(asp1^{D333A}\) cells expressing cen1-GFP (marks chromosome I) \(sad1^-\)-mCherry (marks the spindle pole bodies) (36, 37) revealed several mitotic cells that had an aberrant number of cen1-GFP signals (Fig 5A). \(S.\ pombe\) is a haploid organism, thus during mitosis two segregating cen1-GFP signals representing the two chromosome I sisters are observed (36). In the photomicrographs in Figure 5A, up to 6 cen1-GFP signals were observed suggesting
that these cells were polyploid. We therefore analyzed the ploidy state of wild-type, 
\( asp1^{D333A} \), \( bub3\Delta \), and \( asp1^{D333A} \ bub3\Delta \) strains via FACS analysis (Fig 5B). As 
shown in Figure 5C, \( asp1^{D333A} \) cell populations contain cells with an abnormally high 
DNA content (P2 population). This phenotype is increased in a \( bub3\Delta \) background 
(Fig 5C). P2 cells were longer and wider (on average 15 %) compared to the entire 
cell population.

**Intracellular IP\(_8\) levels are increased in strains without functional Asp1**

Expression of plasmid-encoded \( asp1^{365-920} \) negatively affected intracellular IP\(_8\) levels 
while \( asp1^{365-920/H397A} \) expression had no effect. Consequently one would expect, that 
a strain with an endogenous full-length \( asp1 \) variant with a mutation at position 397 
i.e. \( asp1^{H397A} \) (Fig 6A) generates more IP\(_8\) than a wild-type strain. The HPLC profile 
of the \( asp1^{H397A} \) strain (endogenous \( asp1^{+} \) ORF replaced by \( asp1^{H397A} \) (9)) showed a 
considerable increase of the IP\(_8\) peak compared with the wild-type \( asp1^{+} \) strain (Fig 
6B-D, quantification in 6E).

Next, we investigated the consequences of loss of the entire Asp1 pyrophosphatase 
domain on IP\(_8\) pools. For this we analyzed a strain which expressed an endogenous 
\( asp1 \)-deletion variant consisting only of the Asp1 kinase domain \( asp1^{1-364} \) (Fig 6A). 
The inositol polyphosphate profile of the \( asp1^{1-364} \) strain also showed significantly 
higher IP\(_8\) levels (Fig 6F-G, quantification 6H). The similarity of the inositol 
polyphosphate profiles of the \( asp1^{H397A} \) and \( asp1^{1-364} \) mutant strains demonstrates 
that the change in IP\(_8\) levels observed for the \( asp1^{H397A} \) strain is solely due to the 
missing pyrophosphatase activity.
All conserved residues of the M1 phosphatase motif are essential for enzymatic function

We have shown previously that bacterially produced, recombinant Asp1 protein generated IP$_7$ in vitro using IP$_6$ as a substrate (10). The addition of Asp$_{1-365-920}$ to such a kinase assay reduced the IP$_7$ amount in a dose-dependent manner. However, addition of Asp$_{1-365-920/H397A}$ had no effect demonstrating that this Asp1 pyrophosphatase variant had no enzymatic activity (10). To determine the role of the two other conserved amino acids of the M1 motif, we exchanged the arginine residues R396 and R400 to alanine individually (Fig 7A) and tested the ability of these mutants to dephosphorylate Asp1 kinase generated IP$_7$ in vitro. It had been reported that recombinant bacterially expressed Asp1 is capable of incorporating an iron-sulfur cluster and that the presence of these iron-sulfur clusters inhibits the pyrophosphatase activity (29). Thus, we assessed the content of iron-sulfur clusters for all bacterially produced, recombinant Asp1 variants and found that our protein samples contain no iron-sulfur clusters (Fig S3A).

Recombinant GST-Asp$_{1-365-920/R396A}$, GST-Asp$_{1-365-920/R400A}$, GST-Asp$_{1-365-920}$ and GST-Asp$_{1-365-920/H397A}$ proteins were generated in bacteria and the activity of these four Asp1 variants tested in an in vitro pyrophosphatase assay. First, the IP$_7$ substrate for the assay was synthesized using the Asp1 kinase domain (Asp$_{1-364}$), which was heat-inactivated after the reaction. Second, Asp1 pyrophosphatase variants were added to the mixture and incubated. The inositol polyphosphates present were then analyzed by PAGE (38). As shown previously (10) wild-type Asp$_{1-365-920}$ massively reduced the amount of IP$_7$ (Fig 7B, lane 2 versus input in lane 1) while the presence of Asp$_{1-364-920/H397A}$ did not (Fig 7B, lane 3). Similarly, GST-Asp$_{1-365-920/R396A}$ and GST-Asp$_{1-365-920/R400A}$ were unable to reduce the amount of IP$_7$ in our assay (Fig 7B lanes 5
demonstrating that all conserved residues of the M1 motif were essential for *in vitro* enzymatic activity.

To investigate the *in vivo* function of Asp$^{1365-920/R396A}$ and Asp$^{1365-920/R400A}$, the TBZ sensitivity of a wild-type strain expressing *asp*$_{1365-920/R396A}$ or *asp*$_{1365-920/R400A}$ on a plasmid via the *nmt1*+ promoter was examined. Western blot analysis showed that expression levels of these Asp$^{1365-920}$ variants were similar (Fig S2). In contrast to Asp$^{1365-920}$ neither Asp$^{1365-920/R396A}$ nor Asp$^{1365-920/R400A}$ increased TBZ sensitivity of the strain (Fig 7C).

Previously, we had shown that an *asp1Δ* strain was hypersensitive to TBZ and that this phenotype was rescued by plasmid-borne high-level expression of either wild-type *asp1*+ or *asp1*R396A (10). To analyze if Asp1R400A could rescue the TBZ hypersensitivity of the *asp1Δ* strain, we expressed this *asp1*+ version in the *asp1Δ* strain. However high expression of *asp1*R400A led to cell death by lysis (Fig 7D, data not shown). The molecular basis for the lethality is unclear, however we and others had shown previously that plasmid-borne high expression of *asp1*H397A was also lethal due to cell lysis (2, 10).

Low-level expression of *asp1*R400A did not affect cell growth (Fig 7D). Thus we determined if low-level expression of *asp1*R400A could rescue the inability of the *asp1Δ* strain to switch to pseudohyphal invasive growth (9). This phenotype cannot be rescued by plasmid-encoded wild-type *asp1*+ under low level expression conditions (Fig 7E). However low-level expression of either *asp1*H397A or *asp1*R400A gave rise to invasively growing colonies (Fig 7E and quantification in Fig 7F). We conclude that Asp1R400A is able to generate more IP$_8$ than the wild-type Asp1 protein.
Thus all three conserved amino acids of the histidine acid phosphatase M1 motif RHADR are essential for Asp1 pyrophosphatase activity.

Isoleucine 808 is critical for Asp1 pyrophosphatase function

Histidine acid phosphatases require the presence of an aspartate in the M2 motif HD as proton donor during the enzymatic reaction (28). No aspartate is found at this position in any Vip1 family member; in Asp1 an isoleucine residue is present at this position. To determine if an exchange to aspartate at this position influences pyrophosphatase activity, we assayed if Asp1^{365-920/I808D} (Fig 8A) could dephosphorylate IP$_7$ in our in vitro assay. This was not the case (Fig 8B). Furthermore expression of plasmid-borne asp1^{365-920/I808D} did not cause TBZ hypersensitivity (Fig 8C). To test if intracellular IP$_8$ levels were affected by the mutation at position 808 of Asp1, we constructed a strain in which the endogenous asp1$^+$ gene was replaced by asp1$^{I808D}$. Interestingly, this strain was more resistant to TBZ than a wild-type strain, similar to the asp1$^{H397A}$ strain (Fig 8D) showing that the Asp1$^{I808D}$ pyrophosphatase domain was non-functional.

Using HPLC-based analysis of inositol polyphosphates, we determined cellular IPP levels of the asp1$^{I808D}$ strain. IP$_8$ was increased approximately twofold in the asp1$^{I808D}$ strain compared to a wild-type strain (Fig 8E-G). Therefore, alteration of amino acid 808 of Asp1 to aspartate, which is the proton donor in classical histidine acid phosphatases, abolished pyrophosphatase function.

Finally we analyzed the function of the conserved histidine of the M2 motif (position H807) (Fig 8A). A publication had described this residue to be essential for pyrophosphatase function in vitro (29). However, expression of the asp1$^{365-920/H807A}$
gave rise to TBZ hypersensitivity indicating that this variant was functional (Fig S4A).

We thus tested this variant in our in vitro pyrophosphatase assay. The addition of 8 µg of either Asp1<sup>365-920</sup> or Asp1<sup>365-920/H807A</sup> to the assay reduced IP<sub>7</sub>, while the presence of Asp1<sup>365-920/R400A</sup> had no effect on IP<sub>7</sub> levels (Fig S4B lanes 2-4). This result shows that Asp1<sup>365-920/H807A</sup> still retains pyrophosphatase activity. To understand the discrepancy between our data and those of (29), we repeated the assay with 4 and 2 µg of the relevant proteins. 4 µg of protein led to a partial degradation of the IP<sub>7</sub> input (Fig S4B lanes 5-7) while no pyrophosphatase activity was detected when 2 µg of the proteins were used (Fig S4B lanes 9-11). Thus Asp1<sup>365-920/H807A</sup> retains residual pyrophosphatase activity.

Identification of <i>S. pombe</i> Met10 protein, which inhibits Asp1 pyrophosphatase activity in vitro

Our data show that the Asp1 protein harbors two enzymatic activities of opposing function and that MT stability and the dimorphic switch directly correlate with intracellular IP<sub>8</sub> levels. To find Asp1 interacting proteins that influence the function of the two domains, we conducted an extensive yeast-2-hybrid screen using pGBK7-asp1<sup>+</sup> as bait and an <i>S. pombe</i> cDNA library constructed in the pGAD GH vector (Takara). Out of 2x 10<sup>7</sup> transformants (four-fold coverage of the library), 150 plasmids with putative interacting candidates were isolated and retested. One of the Asp1 interacting proteins was encoded by the uncharacterized ORF <i>SPCC584.01c</i>. which interacted specifically with the Asp1 pyrophosphatase domain (Fig 9A). <i>SPCC584.01c</i> encodes a protein with a predicted size of 111.3 kDa which has 36% overall sequence identity and 52% similarity to <i>Saccharomyces cerevisiae</i> Met10, the
alpha subunit of assimilatory sulfite reductase involved in methionine and cysteine synthesis (39). Due to this similarity the ORF SPCC584.01c was named met10+ in the S. pombe database PomBase and thus we refer to the protein as Met10.

To analyze if the S. pombe Met10 protein had a similar function to that described for S. cerevisiae Met10, we analyzed the growth behavior of an S. pombe met10Δ (deletion of met10+ ORF) strain. The met10Δ strain required cysteine and methionine in the media for growth (Fig 9B) which is also the phenotype of the S. cerevisiae MET10 deletion strain (40). Plasmid-borne overexpression of met10+ was lethal in the wild-type strain (Fig 9C). However, overexpression of met10+ was not lethal in the asp1Δ strain (Fig 9D), indicating that the lethal phenotype requires the presence of Asp1. Thus, asp1+ and met10+ interact genetically. We tried to co-immunoprecipitate Asp1 and Met10 proteins in a strain where the met10+ ORF had been fused with gfp using a GFP antibody followed by western blot analysis with a polyclonal Asp1 antibody (41). However, co-immunoprecipitation using exponentially growing cells was not successful. Thus we used far western blot analysis to determine whether Met10 and Asp1365-920 interact. Recombinantly produced and purified GST-Met10 interacted with His-Asp1365-920, demonstrating that the proteins can bind to each other (Fig 9E).

We next analyzed the subcellular localization of the Met10-GFP protein. Photomicroscopic analysis showed that Met10-GFP was associated with tubular-like structures as has been observed for mitochondria (42). Staining of Met10-GFP cells with the mitochondria specific dye Mitotracker revealed co-localization (Fig 10A). Thus Asp1 can associate with a protein that co-localizes with mitochondria. Interestingly, in a screen for genes needed for survival under oxidative stress conditions numerous genes related to mitochondrial function were identified and the
\(asp1^+\) ORF was one of the candidates (43). Indeed we found that mitochondria distribution depended on functional Asp1 kinase. In \textit{S. pombe} the mitochondria network is comprised of interconnected tubular-like structures that are MT associated (42), which guarantees proper mitochondria positioning and inheritance (44). We found that in an \(asp1\Delta\) strain mitochondrial distribution was abnormal. Visualization of mitochondria via the mitochondria inner membrane protein Cox4-RFP (44) showed that in the \(asp1^+\) or \(asp1^{H397A}\) strain background, 83\% and 96\% of cells showed the normal tubular-like mitochondrial structures (Fig 10B). However, this number was reduced to 43\% in \(asp1\Delta\) cells. Instead these cells had aberrant mitochondrial structures, the most prominent being aggregated mitochondria at the cell end(s) (Fig 10B). This phenotype has been described previously for mutant \textit{mmb1} cells (44). The Mmb1 protein attaches the tubular mitochondria to the MT cytoskeleton (44).

Intriguingly, when we expressed \(asp1\) variants with a functional pyrophosphatase domain in an \textit{mmb1}\(\Delta\) (deletion of \textit{mmb1}\(^+\) ORF) strain on a plasmid, the strains were unable to survive (data not shown). Thus, Asp1-generated IP\(_8\) has a role in mitochondrial function/organization.

To determine if Met10 affects Asp1 pyrophosphatase function, bacterially produced, recombinant GST-Met10 was added to an Asp1\(^{365-920}\) containing \textit{in vitro} pyrophosphatase assay. As the \textit{S. cerevisiae} Met10 protein interacts with the cytoplasmic iron-sulfur assembly (CIA) component Mms19 (alias Met18) that is required for Fe-S protein maturation and is also a target of this complex, we first determined if the recombinant GST-Met10 protein contained an iron-sulfur cluster (45). This was not the case (Fig S3B).

As shown previously, the presence of GST-Asp1\(^{365-920}\) in the pyrophosphatase assay resulted in dephosphorylation of IP\(_7\) (Fig 10C, lane 2). However, in the presence of
equimolar amounts of GST-Met10 and GST-Asp\(^{365-920}\) in the assay, IP\(_7\) was not
dephosphorylated (Fig 10C, lane 3). Thus, Met10 inhibits the function of the Asp1
pyrophosphatase domain. As both proteins were GST tagged and GST-GST
interactions can occur, we repeated the assay using Asp\(^{365-920}\)-His and GST-Met10.
Again, Asp\(^{365-920}\)-His dephosphorylated IP\(_7\) but not in the presence of GST-Met10
(Fig 10C, lanes 4 and 5). Thus, \textit{in vitro} the Met10 protein is an inhibitor of the Asp1
pyrophosphatase activity. To determine, whether the inhibitory effect of Met10 was
specific for Asp1 pyrophosphatase, we tested if Met10 could inhibit another protein
with pyrophosphatase activity. For this purpose recombinant GST-Ddp1 was
generated and used in our \textit{in vitro} assay. The \textit{S. cerevisiae} Ddp1 protein has inositol
pyrophosphatase activity (25). Ddp1 enzymatic activity dephosphorylated IP\(_7\) (Fig
10C, lane 6 and 7) and this ability was not altered in the presence of equimolar
amounts of GST-Met10 (Fig 10C, lane 7). Thus, \textit{in vitro} Met10 inhibits specifically
Asp\(^{365-920}\) pyrophosphatase activity. However as the inositol polyphosphate profiles
of wild-type and \textit{met10A} strains were similar, loss of Met10 was not sufficient to
significantly down-regulate Asp1 pyrophosphatase activity \textit{in vivo} (data not shown).
Discussion

In this work, we have established that Asp1 is a bi-functional enzyme *in vivo* responsible for the synthesis and hydrolysis of one specific inositol pyrophosphate: IP<sub>8</sub>. Functional dissection of the Asp1 pyrophosphatase by mutational analysis combined with our previous analysis of Asp1 function demonstrated that morphogenesis and chromosome transmission are regulated by IP<sub>8</sub> in a dose dependent manner (9, 10, 12). In fact a direct correlation exists for the optimization of a cellular process and IP<sub>8</sub> levels: for example, higher-than-wild-type IP<sub>8</sub> levels resulted in higher-than-wild-type chromosome transmission fidelity. On the other hand, strains with less-than-wildtype or no IP<sub>8</sub> showed decreased chromosome transmission fidelity (12). The output of the Asp1 kinase is counter steered by the Asp1 pyrophosphatase, thus up- or down-regulation of pyrophosphatase activity controls intracellular IP<sub>8</sub> levels.

Identification of conserved amino acids essential for pyrophosphatase function

We were the first to show in an *in vitro* assay that a member of the PPIP5Ks/Vip1 family proteins has pyrophosphatase activity: IP<sub>7</sub> produced by the Asp1 kinase was reduced by Asp1<sup>365-920</sup> demonstrating that the C-terminal Asp1 domain was enzymatically active (10). Pyrophosphatase activity depended on the two conserved signature motifs of histidine acid phosphatases M1 and M2. The conserved amino acids of M1 were essential for enzymatic function of the Asp1 pyrophosphatase *in vitro* and *in vivo*. Similarly, our *in vivo* read-out assays for strains expressing *asp1* variants with the mutation R396A or R400A imply that these are also
pyrophosphatase negative (10). In metazoans, a mutation in either PPIP5K protein complementary to the Asp1^{R396} mutation had a similar effect (46).

Of particular interest was the second amino acid of the M2 motif HD as this amino acid is not conserved in PPIP5Ks/Vip1 family members (1). For Asp1 the M2 motif is HI. The catalytic mechanism of histidine acid phosphatases requires a proton donor, which is typically a glutamate or aspartate residue proximal to the active site (28). Replacement of the glutam ate/aspartate residue resulted in a dramatic decrease of enzymatic activity (47, 48). Thus it was of great interest to determine the enzymatic activity of a mutant Asp1 variant where the wild-type isoleucine had been replaced by aspartate resulting in the “perfect” M2 signature motifs of histidine acid phosphatases. Asp1^{I808D} variants had no \textit{in vitro} and \textit{in vivo} pyrophosphatase activity. Furthermore, replacement of isoleucine 808 by valine, which is found at this position in metazoan PPIP5Ks/Vip1 family members also led to inactivation of pyrophosphatase function (data not shown) (1, 3).

Finally, the histidine in the M2 motif is conserved in histidine acid phosphatases and all PPIP5Ks/Vip1 family members (1). A previous publication showed that mutation of this residue generating Asp1^{397-920/H807A} led to a loss of about 95% activity \textit{in vitro} (29). However, we found that Asp1^{365-920/H807A} retained residual pyrophosphatase activity. The different results obtained might be due to a different experimental set-up. Interestingly, it has been shown for the rat fructose 2,6 bisphosphatase that the replacement of the equivalent histidine did not significantly change the enzymatic activity (49).

\textbf{Cellular levels of IP_3 are regulated by Asp1 pyrophosphatase activity}
Ectopic expression of \( \text{asp}^{365-920} \) massively reduced cellular IP\(_8\) amounts while endogenous pyrophosphatase-dead variants increased cellular IP\(_8\) levels. Thus, intracellular IP\(_8\) levels can be up- or down-regulated by the enzymatic activity of the Asp1 pyrophosphatase domain. These high energy molecules are generated solely by the Asp1 kinase domain as \( \text{asp}1\Delta \) and \( \text{asp}1^{D333A} \) strains had no-detectable IP\(_8\) (2, 9, 10). Similarly, \( S. \) \textit{cerevisiae} and \( C. \) \textit{neoformans} strains with a deletion of the gene, which encodes the PPIP5Ks/Vip1 protein, have no or massively reduced IP\(_8\) levels but elevated IP\(_7\) levels implying that in these organisms PPIP5Ks/Vip1 proteins generate IP\(_8\) (6, 11, 20). The \textit{in vivo} function of the pyrophosphatase domain of PPIP5Ks/Vip1 proteins in other organisms remains to be studied.

\textbf{The Asp1 interacting protein Met10 inhibits the pyrophosphatase activity \textit{in vitro}}

We identified the mitochondria-associated Met10 protein that specifically interacted with the Asp1 pyrophosphatase domain and inhibited its function \textit{in vitro}. Met10 belongs to a conserved protein family involved in the methionine biosynthesis pathway. Interestingly, the \( S. \) \textit{cerevisiae} Met10 member interacts physically with the highly conserved Mms19 (alias Met18) protein (45). Mms19, which was identified previously to be also required for methionine biosynthesis, has since been shown to be a member of the Fe-S protein assembly (CIA) machinery (45, 50, 51). Incorporation of iron-sulfur clusters into proteins is mediated by a two-step mechanism occurring in the mitochondria and the cytosol (reviewed in (52)). Mms19 serves as part of a CIA targeting complex responsible for iron-sulfur cluster insertion into proteins involved in specific cellular processes including methionine biosynthesis (45). Mms19 is needed for the sulfite reductase activity of the \( S. \) \textit{cerevisiae} Met5-
Met10 complex where Met10 represents a Fe-S containing protein (45). As the Asp1
pyrophosphatase activity is inhibited by the incorporation of an iron-sulfur cluster in
vitro (29), it is possible that such an iron-sulfur cluster transfer could occur via S.
pombe Met10 in vivo. However, the consequences of such a transfer in vivo remain
unclear. Inositol polyphosphate profiles of wild-type and met10Δ strains were
comparable and expression of an asp1 variant where one of the cysteine residues
required for binding the iron-sulfur cluster was mutated (29), had no phenotypic
consequences for yeast cell growth under varying conditions (data not shown).

**IP₈ and its impact on the microtubule cytoskeleton**

The human MMS19 protein is part of the 5 component MMXD complex required for
chromosome transmission fidelity. MMS19 localizes to the mitotic spindle and a
knockdown of MMS19 gave rise to highly abnormal spindles (53). Thus, MMS19 is
required for spindle formation/function. We have previously shown that S. pombe
Asp1 kinase function controls bipolar spindle formation by modulating in- and
outward pulling forces at the spindle (12). Our results raise the intriguing possibility
that IP₈ modulated MT regulation might involve the Met10-Mms19 pathway. Although
the impact of the Mms19 protein on the MT cytoskeleton has not been tested in S.
pombe, it has been found that S. pombe cells with a deletion of the mms19⁺
encoding gene have an abnormal cell shape showing branched and curved cells
(54). Such cell shapes are indicative of a defective interphase MT cytoskeleton
(reviewed in (55)). Furthermore a S. cerevisiae met10Δ bim1Δ double mutant strain
is non-viable (56). Bim1 is a part of the EB1 family, which represents a central
element of polymerizing MT plus-ends (57). Thus, it is feasible that the Met10 and
Mms19 proteins play a role in MT modulation.
Central elements in bipolar spindle assembly/function and segregation of spindle poles are kinesin-5 family members (58). The human kinesin-5 Eg5 protein has been in the focus of research due to its important role in tumorigenesis. This motor protein is up-regulated in many types of cancer such as pancreatic cancer, is associated with poor prognosis and can trigger genome instability in the mouse system (34, 35, 59). It is thus of great interest that intracellular IP₈ levels control spindle association of the S. pombe kinesin-5 Cut7. This finding raises the exciting possibility that IP₈ levels could be used as a tool to control Eg5 up-regulation.
Materials and Methods

Strains, plasmids and media

All strains used are listed in Table 1. Generation of \textit{asp1} mutant strains was performed as described (9). Gene deletions and ORF fusions to \textit{gfp} were done by PCR-based gene targeting (60) using the kanamycin resistance (kan\(^R\)) cassette. 

\textit{asp1}\(^+\), \textit{asp1}\^{1-364}, \textit{asp}\^{365-920} plasmids are derivatives of pJR2-3XL (9, 12, 61). For the \textit{asp1}\^{365-920/H397A}, \textit{asp1}\^{365-920/H807A}, \textit{asp1}\^{365-920/R396A}, \textit{asp1}\^{365-920/R400A}, \textit{asp1}\^{365-920/I808D} containing plasmids, PCR fragments were generated by directed mutagenesis using the QuikChangeII Site-Directed Mutagenesis Kit (Agilent Technologies) and cloned into pJR2-3XL (61) via homologous recombination in \textit{S. cerevisiae} (62).

\textit{S. pombe} strains were grown in rich media (YE5S) or minimal media (MM) with supplements (63). To control the \textit{nmt1}\(^+\) promoter, cells were grown in MM with or without 5 \(\mu\)g/ml thiamine. Experiments were carried out at 25\(^\circ\)C, except the invasive growth experiments and the labeling with \(^{3}\text{H}\)inositol which were performed at 30\(^\circ\)C. Microscopy was performed at temperatures stated in the respective figure legends.

Western blot analysis

Transformants with plasmid-borne expression of \textit{asp1} variants were grown under plasmid selective conditions without thiamine for 24 h at 25\(^\circ\)C before protein extraction. Protein extraction was carried out as described (9) using an anti-GFP antibody when \textit{asp1} variants were fused to \textit{gfp} (monoclonal mouse; Roche) or using an anti-Asp1 antibody (41) and an anti-\(\gamma\)-tubulin antibody (monoclonal mouse; Sigma).
**In vitro enzymatic activity of Asp1 variants**

Recombinant proteins Asp1\(^{1-364}\) and Asp1\(^{365-920}\) were previously described (10). 1751 bp PCR fragments containing asp1\(^{365-920/R396A}\), asp1\(^{365-920/H397A}\), asp1\(^{365-920/R400A}\), asp1\(^{365-920/H807A}\), asp1\(^{365-920/I808D}\), 3101 bp fragment containing the entire met10\(^{+}\) ORF or 649 bp fragment containing the entire ScDDP1 ORF were cloned into E. coli expression vector pKM36 to generate GST-tagged proteins or into E. coli expression vector pFT25 to generate His-tagged proteins. Proteins were expressed and purified from E. coli Rosetta (DE3) strain according to protocol (Sigma Aldrich).

Enzymatic reactions were performed as described (10, 38). For the kinase reaction, 4 µg of purified Asp1\(^{1-364}\) protein was incubated for 16 h at 37°C with 300 µM IP\(_6\) (Sigma-Aldrich) followed by Asp1\(^{1-364}\) inactivation (65°C for 20 min). Inactivation was verified by performing a kinase assay with the treated Asp1\(^{1-364}\) protein. 30 µl of the generated IP\(_7\) were incubated with 8 µg of Asp1\(^{365-920}\) variants for 16 h at 37°C, followed by PAGE analysis. In Fig 10C, 8 µg of GST-Met10, 2 µg of GST-Ddp1 and 4 µg of Asp1\(^{365-920}\) were used in the assay.

**[^3H]inositol labeling and HPLC analysis.**

[^3H]inositol labeling of *S. pombe* cultures was performed as described (31). Cells were grown overnight at 30°C in MM with 55 µM inositol followed by dilution to OD\(_{600}\) of 0.05 in 5 ml MM with 10 µM inositol supplemented with 6 µCi/ml of [^3H]inositol and incubated until OD\(_{600}\) reached 0.8-1.6 (30 to 48 h). Extraction of inositol polyphosphates was performed as described (31) and resolved by anion exchange chromatography HPLC (using the partisphere SAX 4.6 × 125 mm column; Whatman).
Collected fractions were analyzed by scintillation counting. Soluble inositol polyphosphate levels were normalized against total lipid inositol content. Statistics for the ratios of IP₈/ IP₆, IP₈/IP₇ and IP₇/ IP₆ were performed using Graphpad Prism 5.

Electronic absorption spectroscopy

Electronic absorption spectroscopy was used to determine the iron-sulfur cluster content of Asp1³⁶⁵⁻⁹²⁰. Electronic absorption spectra were recorded using a double-beam JASCO V-650 spectrophotometer at room temperature. Spectra were obtained using a 1 cm path length cuvette for samples with a protein concentration of ~1 µg/ml.

Flow cytometry

Yeast flow cytometry was carried out as described using Sytox green (64) and a FACS Aria (BD Biosciences). 10000 cells were counted/sample and all strains were counted at least twice and were grown at different temperatures before fixation (20-36 °C). The data shown in Fig 5B-C was obtained from cells incubated at 30 °C but is representative for all other temperatures. DNA content of cells was defined using the temperature sensitive cdc11-123 strain as a standard (65).

Invasive growth assay

Transformants were grown overnight in plasmid selective media with or without thiamine. Cells were diluted to an end concentration of 2x 10⁶ cells/ml and 5 µl of cells were patched on plasmid selective agar plates at equal distance from each other. Incubation was done at 30°C for 21 days (66). To analyze invasive growth,
surface grown cells were removed by washing, plates were dried and then photographed using a binocular microscope and digital Sony DSLR camera. Quantification of invasive growth was done by determining the number of invasive colonies per mutant in 3 different transformants in at least 3 different experiments.

Yeast 2-hybrid screen

Yeast-2-hybrid screen was performed using the AH109 strain transformed with pGBK7-asp1+ as bait and mated with Y187 transformed with an S. pombe cDNA library constructed in the pGAD GH vector (MATCHMAKER cDNA Library (XL4000AA Takara). Mating was plated on SD-Leu-Trp-His and incubated for 8 days. Plasmids from positive candidates were co-transformed with pGBK7-asp1+ into strain AH109 and further analyzed.

Microscopy

Live-cell imaging was performed using a Zeiss spinning-disk confocal microscope equipped with a Rolera EM-C (QImaging) camera. Transformants expressing cut7+::GFP were pre-grown for 20 h at 30°C in plasmid selective media. Videos were taken at 30°C. For asp1+ cut7+::GFP and asp1D333A cut7+::GFP strains growth and imaging was done at 33°C. A maximum intensity projection (MIP) picture (25 z-slices (transformants) or 35 z-slices (strains) in 0.5 µm intervals) of the time point with the strongest fluorescence signal on a short spindle was generated and used for analysis. Analysis was performed using Zen2012 and Axiovision software. Image processing was done with Canvas 14 and Adobe Photoshop CS2. Intensity of GFP fluorescence signals was measured via ImageJ 1.44 (NIH). The asp1D333A bub3Δ
strain was pre-grown for 24 h at 30°C. Shown in Fig 5A are MIP images of a single cell. For live-cell imaging of met10\(^{+}\)-GFP expressing cells stained with Mitotracker or cox4\(^{+}\)-RFP cells expressing different asp1 variants, cells were recorded at 25 °C with a z-stack of 25 z-slices with a distance of 0.5 μm and a MIP image generated.

Statistics for fluorescence signal intensity ratios and spindle break frequencies were performed using Graphpad Prism 5.

**Far-Western blot analysis**

GST-Met10 and Asp\(^{365-920}\)-His were purified from the *E. coli* Rosetta (DE3) strain. 1 μg of GST-Met10 or Asp\(^{365-920}\)-His (prey proteins) were separated by 10% SDS-PAGE and then transferred to a PVDF membrane. After denaturation with 6 M guanidine-HCl, the prey protein was gradually renatured on the membrane by incubation with decreasing concentrations of guanidine-HCl in a buffer containing Glycerin 10%, 0.1 M NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 0.1 % Tween-20, 2% milk and 1 mM DTT. After an overnight incubation at 4°C with the buffer containing no guanidine-HCl, 10 μg/ml of Asp\(^{365-920}\)-His, GST-Met10 or GST (bait proteins) were incubated 5 h at RT with the regenerated membrane. After 3 washes with PBS, protein interactions were detected using His (Roche) or GST (Thermo Fisher) antibodies.

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References


is activated when cells are exposed to hyperosmotic stress. J Biol Chem 282:30763-75.


Figure Legends

Fig 1. Asp1 kinase generates IP₈.

A to C: HPLC elution profiles of inositol polyphosphates of wild-type (WT), asp₁^{D333A} and asp₁Δ strains. *S. pombe* cells were radiolabeled with [³²P]inositol and cell lysates separated using anion-exchange HPLC. CPM: counts per minute. D: Left: diagrammatic representation of IP₈ levels relative to IP₆. Right: diagrammatic representation of IP₇ levels relative to IP₆. (WT: n= 3; asp₁^{D333A} n= 2; asp₁Δ n= 3. ***: P ≤ 0.001; *: P ≤ 0.05, t-test). The fold-change of IP₈/ IP₆ is as follows: (WT set at 1.00); 0.12 (asp₁^{D333A}) and 0.11 (asp₁Δ). Fold-change of IP₇/ IP₆: 6.26 (asp₁^{D333A}) and 4.56 (asp₁Δ).

Fig 2. In vivo analysis of Asp₁^{365-920} and Asp₁^{365-920/H397A} function.

A: Diagrammatic representation of the dual-domain structure of Asp1 with kinase (K, black box) and pyrophosphatase (P, light grey box) regions. Enlargement of pyrophosphatase domain with the signature motifs M1 and M2 of histidine acid phosphatases (dark grey boxes) (1). In Asp1, the aspartate residue of M2 is replaced by isoleucine (HI instead of HD). B: Serial dilution patch tests (10⁴ to 10¹ cells) of a wild-type strain transformed with vector (control) or plasmids expressing asp₁^{365-920} or asp₁^{365-920/H397A} from the thiamine-repressible promoter nmt1⁺. Transformants were grown under plasmid selective conditions in absence or presence of 7 µg/ ml
TBZ at 25°C for 7 days. C: Invasive growth assay. Left: A total of $10^5$ wild-type cells transformed with either vector control or plasmids with $asp^{1365-920}$ or $asp^{1365-920/H397A}$ were spotted on plasmid selective medium without thiamine and incubated for 21 days at 30°C (top panels, surface growth). Plates were washed and all surface growth rubbed off. Invasively growing colonies remained (bottom panels) and were counted. Quantification shown on the right: 3 transformants were analyzed per plasmid in triplicate, ns= not significant, ***: $P < 0.0005$, t test. The number of agar-invading colonies of the $asp^{1365-920/H397A}$ transformants and the control transformants were $16.5 \pm 4.0$ and $17.5 \pm 3.6$, respectively.

**Fig 3. Asp1^{365-920} has pyrophosphatase activity *in vivo*.**

A to C: HPLC elution profiles of inositol polyphosphates of the wild-type strain transformed with (A) vector control or $asp^{1365-920}$ or $asp^{1365-920/H397A}$ expressing plasmids (B and C, respectively). Cells were radiolabeled with $[^3H]$ inositol and cell lysates separated using anion-exchange HPLC. D: Diagrammatic representation of IP$_8$ levels relative to IP$_6$ (left) and IP$_7$ levels relative to IP$_6$ (right) normalized to the vector control using data from A, B and C. (control: $n=4$; $asp^{1365-920}$ $n=4$; $asp^{1365-920/H397A}$ $n=4$. **: $P \leq 0.01$; *: $P \leq 0.05$; ns: not significant, t-test). The fold-change of IP$_8$/ IP$_6$ is as follows: (control set at 1.00); 0.4 ($asp^{1365-920}$) and 5.3 ($asp^{1365-920/H397A}$). Fold-change of IP$_7$/ IP$_6$: 9.3 ($asp^{1365-920}$) and 1.8 ($asp^{1365-920/H397A}$). E: MT stability and the dimorphic switch require intracellular IP$_8$, which are down-regulated by Asp1 pyrophosphatase activity.

**Fig 4. IP$_8$ controls Cut7-GFP spindle association.**
A: Photomicrographs of cut7−gfp cells transformed with a vector control or an asp1365-920 expressing plasmid. Scale bar= 2 μm. B: Quantification of the fluorescence signal of Cut7-GFP on short spindles. For relative signal intensity at the spindle midzone compared to the spindle ends, the fluorescence signal at the midzone was normalized against the background (square 5 – square 6) and divided by the fluorescence intensity at spindle ends (square 1 – square 2 and square 3 – square 4). C: Diagrammatic representation of the ratios spindle midzone/spindle ends (control: n= 29; pasp1365-920 n= 24; ***: P ≤ 0.001, t-test; significant outliers removed using Grubbs’ test). D: Diagrammatic representation of the frequency of spindle breaks in the indicated transformants (control: n= 30; pasp1365-920 n= 29; ***: P ≤ 0.001, χ²-test). E: Diagrammatic representation of the ratios spindle midzone/spindle ends (control: n= 30; pasp1365-920 non-breaking n= 23; pasp1365-920 breaking n= 17 (9 cells); ***: P ≤ 0.001, *: P ≤ 0.05, t-test). F: Diagrammatic representation of the ratios spindle midzone/spindle ends (asp1+ cut7-GFP: n= 29; asp1D333A cut7-GFP: n= 24; ***: P ≤ 0.001, t-test; significant outliers removed using Grubbs’ test). Analysis was carried out at 33 °C.

Fig 5. asp1D333A cell population contains polyploid cells.

A: Photomicrographs of a mitotic asp1D333A bub3Δ cell expressing sad1−-mCherry and cen1-GFP. Time between images: 1 min. Scale bar= 2 μm. 2/11 analyzed asp1D333A bub3Δ double mutant cells showed this phenotype. B: FACS analysis of the indicated strains. Cells were gated for size revealing that cell populations with an asp1D333A background were much more heterogenous than asp1+ populations. The P2 area contains the largest cells. C: Measurement of DNA content (2-32N) of the
indicated cell population; left: entire population; right: P2 population. DNA content of peaks was defined by using the cdc11-123 strain as standard (Suppl. Fig 5) (65).

**Fig 6. Loss of functional Asp1 pyrophosphatase domain results in increased IP₈ levels.**

**A:** Diagrammatic representation of Asp1 variants analyzed. All variants were expressed from the endogenous asp1⁺ locus. **B** and **C:** HPLC elution profile of inositol polyphosphates of the wild-type type (WT) or asp1¹⁴[H₃⁹⁷A] strain. **D:** Comparison of part of the inositol pyrophosphate profiles of the wild-type and asp1¹⁴[H₃⁹⁷A] strains. **E:** Diagrammatic representation of IP₈ levels relative to IP₇. (WT: n= 4; asp1¹⁴[H₃⁹⁷A] n= 3; *: P ≤ 0.05, t-test). The fold-change of IP₈/ IP₇ is 2.81 higher for the asp1¹⁴[H₃⁹⁷A] strain compared to the wild-type strain. **F:** HPLC elution profile of inositol polyphosphates of the asp1¹⁻₃⁶⁴ strain. **G:** Comparison of inositol pyrophosphate profiles of the wild-type and asp1¹⁻₃⁶⁴ strains (data used for this wild-type were obtained from a strain grown in parallel to the asp1¹⁻₃⁶⁴ strain) and **H:** Diagrammatic representation of IP₈ levels relative to IP₇ and normalized to the wild-type. (WT: n= 4; Asp1¹⁻₃⁶⁴ n= 3; **: P ≤ 0.01, t-test). The fold-change of IP₈/ IP₇ is 1.67 higher for the asp1¹⁻₃⁶⁴ strain compared to the wild-type strain.

**Fig 7. The conserved amino acids of the M1 motif are essential for pyrophosphatase activity.**

**A:** Diagrammatic representation of Asp1 pyrophosphatase M1 motif mutants. **B:** In vitro pyrophosphatase assay with using Asp1¹⁻³⁶⁵⁻⁹²⁰, Asp1³⁶⁵⁻⁹²⁰[H₃⁹⁷A], Asp1³⁶⁵⁻⁹²⁰[R₄⁰⁰A] or Asp1¹⁻³⁶⁵⁻⁹²⁰[R₃⁹⁶A]. 8 µg of the indicated proteins were added to Asp1 kinase
generated IP$_7$ (input shown in lane 1), incubated for 16 h and the resulting inositol polyphosphates resolved on a 35.5% PAGE and stained with Toluidine Blue; - component not added, + component added. All pyrophosphatase variants were tested at least twice in the in vitro assay. C: Serial dilution patch tests (10$^4$ to 10$^1$ cells) of a wild-type strain transformed with vector (control) or plasmids expressing the indicated asp1 variants via the nmt$t^+$ promoter. Transformants were grown under plasmid selective conditions with or without thiamine and with or without 7 µg/ml TBZ at 25°C for 7 days. D: Serial dilution patch tests (10$^4$ to 10$^1$ cells) of an asp1Δ strain transformed with vector (control) or plasmids expressing asp1$^+$ or asp1$^{R400A}$ from the nmt$t^+$ promoter. Transformants were grown as in C. E: Invasive growth assay. Left: 10$^5$ wild-type cells transformed with vector control, asp1$^+$, asp1$^{H397A}$ or asp1$^{R400A}$ plasmids were grown on plasmid selective thiamine-plus medium for 21 days at 30°C (top panels, surface growth). Removal of surface growth by washing revealed invasively growing colonies (bottom panels). F: Quantification of invasively growing colonies; per plasmid 3 transformants were analyzed in triplicate, ***$P$ < 0.0005, t-test. Number of invasive colonies: 81 ± 6 for asp1$^{H397A}$ and 113 ± 8 for asp1$^{R400A}$.

Fig 8. The I808D mutation abolishes Asp1 pyrophosphatase activity

A: Diagrammatic representation of M2 motif mutants. B: In vitro pyrophosphatase assay using 8 µg protein of the indicated Asp1 variants. Assay performed as in Fig. 7B. The pyrophosphatase variants were tested at least twice in the in vitro assay. C: Serial dilution patch tests (10$^4$ to 10$^1$ cells) of a wild-type strain transformed with vector (control) or plasmids expressing asp1$^{365-920}$ or asp1$^{365-920/I808D}$ via nmt$t^+$. Transformants were grown under plasmid selective conditions with or without thiamine and with or without 7 µg/ml TBZ at 25°C for 7 days. D: Serial dilution patch
tests (10⁴ to 10¹ cells) of wild-type, \( \textit{asp1} \Delta \), \( \textit{asp1}^{H397A} \) and \( \textit{asp1}^{I808D} \) strains grown on YE5S full media at 25°C for 5 days with or without 12 µg/ml TBZ. \( \textbf{E:} \) HPLC elution profile of inositol polyphosphates of the \( \textit{asp1}^{I808D} \) strain. \( \textbf{F:} \) Comparison of inositol pyrophosphate profiles of wild-type and \( \textit{asp1}^{I808D} \) strains. \( \textbf{G:} \) IP₈ levels relative to IP₇ and normalized to the wild-type from data shown in F. (WT: n= 4; \( \textit{Asp1}^{I808D} \) n= 3; *: P ≤ 0.05, t-test). The fold-change of IP₈/ IP₇ is 2 for the \( \textit{asp1}^{I808D} \) strain compared to the wild-type strain.

\( \textbf{Fig 9. Characterization of the Asp1 interaction partner Met10.} \)

\( \textbf{A:} \) Yeast-two-hybrid analysis of the interaction between Asp1 and Met10 (SPCC584.01c). \( \textit{S. cerevisiae} \) strain AH109 was co-transformed with a plasmid expressing \( \textit{asp1}^{+} \) fused to the \( \textit{GAL4} \) binding domain (pGBKT7) and a plasmid expressing \( \textit{met10} \) variant (aa 544-1006) fused to the \( \textit{GAL4} \) activation domain (pGADT7). Cells were spotted on plasmid selective SD medium with or without histidine and incubated for 5 days at 30°C. \( \textbf{B:} \) Growth of wild-type and \( \textit{met10} \Delta \) strains on the indicated media: full media (YE5S), minimal media (MM), MM plus 330 µM cysteine (MM+Cys), MM plus 140 µM methionine (MM+Met) or MM plus cysteine and methionine (MM+Cys+Met). \( \textbf{C:} \) Serial dilution patch tests (10⁴ to 10¹ cells) of a wild-type strain transformed with vector (control) or plasmids expressing \( \textit{asp1}^{+} \) or \( \textit{met10}^{+} \) from the \( \textit{nmt1}^{+} \) promoter. Transformants were grown at 25°C for 8 days. \( \textbf{D:} \) Serial dilution patch tests (10⁴ to 10¹ cells) of transformed \( \textit{asp1} \Delta \) cells with vector (control) or plasmids expressing \( \textit{asp1}^{+} \) or \( \textit{met10}^{+} \) via \( \textit{nmt1}^{+} \). Incubation at 25°C for 11 days. \( \textbf{E:} \) Far western analysis. Far left: Coomassie stained gel of 1 µg of the indicated purified proteins. Left: Protein-protein interaction of GST-Met10 (blotted
protein; 138 kDa, arrow) and Asp1\textsuperscript{365-920}-His (probe protein). Detection of GST-Met10 via His antibody. Middle: Control; Asp1\textsuperscript{365-920}-His (blotted protein) and GST (probe protein) using a GST antibody. Right: Protein-protein interaction of Asp1\textsuperscript{365-920}-His (blotted protein; 65 kDa, arrow) and GST-Met10 (probe protein). Detection of Asp1\textsuperscript{365-920}-His via GST antibody. 1 µg of protein was loaded on the gel in all cases.

Concentration of probe proteins: 10 µg/ml.

**Fig 10.** The mitochondrial associated Met10 protein inhibits Asp1 pyrophosphatase activity *in vitro*.

**A:** Live cell imaging of Met10-GFP cells stained with Mitotracker. Shown are maximum-intensity-projection images of interphase cells grown at 25°C. Bar, 10 µm.

**B:** Top: Live cell imaging of the mitochondrial protein Cox4-RFP in *asp1*\textsuperscript{+} or *asp1Δ* cells. Shown are maximum-intensity-projection images of interphase cells grown at 25°C. # normal and * abnormal mitochondrial distribution. Bar, 10 µm. Bottom: quantification of mitochondrial distribution: *asp1*\textsuperscript{+} strain, n = 143; *asp1Δ*\textsuperscript{+397A} strain, n = 77; *asp1Δ* strain, n=44; P** < 0.01, P*** < 0.001 χ2 test). **C:** *In vitro* pyrophosphatase assay: input controls (lanes 1 and 8), 4 µg GST-Asp1\textsuperscript{365-920} (lane 2), 4 µg GST-Asp1\textsuperscript{365-920} plus 6 µg Met10 (lane 3). *In vitro* pyrophosphatase assay using 4 µg Asp\textsuperscript{365-920,-His} (lane 4) or 4 µg Asp\textsuperscript{365-920,-His} plus 8 µg Met10 (lane 5). *In vitro* pyrophosphatase assay using 2 µg Ddp1-GST (lane 6) or 2 µg Ddp1-GST plus 6 µg Met10 (lane 7). Lane 9 shows addition of 2 µg GST. *In vitro* pyrophosphatase assay using 6 µg GST-Met10 and 2 µg GST (lane 10). *In vitro* pyrophosphatase assays involving Met10 protein were repeated 4 times. All assays were incubated for 16 h and the resulting inositol polyphosphates resolved on a 35.5% PAGE and stained.
with Toluidine Blue; - component not added, + component added. Size of proteins used in C: GST-Asp1: ~91 kDa, GST-Met10: ~138 kDa, Asp1^{365-920}.His: ~66 kDa, GST-Ddp1: ~48 kDa.

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Table 1. Strains used in this study.

<table>
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<tr>
<th>S. pombe</th>
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**S. cerevisiae**

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**E. coli**

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Fig 1. Asp1 kinase generates IP₈.

A to C: HPLC elution profiles of inositol polyphosphates of wild-type (WT), asp₁^{D333A} and asp₁Δ strains. *S. pombe* cells were radiolabeled with [³H]inositol and cell lysates separated using anion-exchange HPLC. CPM: counts per minute. D: Left: diagrammatic representation of IP₈ levels relative to IP₆. Right: diagrammatic representation of IP₇ levels relative to IP₆. (WT: n= 3; asp₁^{D333A} n= 2; asp₁Δ n= 3. ***: P ≤ 0.001; *: P ≤ 0.05, t-test). The fold-change of IP₈/ IP₆ is as follows: (WT set at 1.00); 0.12 (asp₁^{D333A}) and 0.11 (asp₁Δ). Fold-change of IP₇/ IP₆: 6.26 (asp₁^{D333A}) and 4.56 (asp₁Δ).
Figure 2

A. Schematic representation of the Asp1 protein with its domains and mutations.

B. Phenotypic analysis of wild-type strain under different conditions:
- + thiamine (low expression)
- - thiamine (high expression) - TBZ
- + TBZ

C. Visual comparison of surface growth and invasive growth for different strains:
- Control
- pasp1^365-920
- pasp1^365-920/H397A

Graph showing invasive growing colonies for different conditions.

*** Indicates statistical significance.
Fig 2. **In vivo analysis of Asp1^{365-920} and Asp1^{365-920/H397A} function.**

A: Diagrammatic representation of the dual-domain structure of Asp1 with kinase (K, black box) and pyrophosphatase (P, light grey box) regions. Enlargement of pyrophosphatase domain with the signature motifs M1 and M2 of histidine acid phosphatases (dark grey boxes) (1). In Asp1, the aspartate residue of M2 is replaced by isoleucine (HI instead of HD). B: Serial dilution patch tests (10^4 to 10^1 cells) of a wild-type strain transformed with vector (control) or plasmids expressing asp1^{365-920} or asp1^{365-920/H397A} from the thiamine-repressible promoter nmt1. Transformants were grown under plasmid selective conditions in absence or presence of 7 µg/ml TBZ at 25°C for 7 days. C: Invasive growth assay. Left: A total of 10^5 wild-type cells transformed with either vector control or plasmids with asp1^{365-920} or asp1^{365-920/H397A} were spotted on plasmid selective medium without thiamine and incubated for 21 days at 30°C (top panels, surface growth). Plates were washed and all surface growth rubbed off. Invasively growing colonies remained (bottom panels) and were counted. Quantification shown on the right: 3 transformants were analyzed per plasmid in triplicate, ns = not significant, ***: P < 0.0005, t test. The number of agar-invading colonies of the asp1^{365-920/H397A} transformants and the control transformants were 16.5±4.0 and 17.5±3.6, respectively.
Fig 3. Asp$^{1365-920}$ has pyrophosphatase activity in vivo.

A to C: HPLC elution profiles of inositol polyphosphates of the wild-type strain transformed with (A) vector control or asp$^{1365-920}$ or asp$^{1365-920/H397A}$ expressing plasmids (B and C, respectively). Cells were radiolabeled with [$^{3}$H] inositol and cell lysates separated using anion-exchange HPLC. D: Diagrammatic representation of IP$_{8}$ levels relative to IP$_{6}$ (left) and IP$_{7}$ levels relative to IP$_{6}$ (right) normalized to the vector control using data from A, B and C. (control: n= 4; pasp$^{1365-920}$ n= 4; pasp$^{1365-920/H397A}$ n= 4. **: P ≤ 0.01; *: P ≤ 0.05; ns: not significant, t-test). The fold-change of IP$_{8}$/ IP$_{6}$ is as follows: (control set at 1.00); 0.4 (pasp$^{1365-920}$) and 5.3 (pasp$^{1365-920/H397A}$). Fold-change of IP$_{7}$/ IP$_{6}$: 9.3 (pasp$^{1365-920}$) and 1.8 (pasp$^{1365-920/H397A}$). E: MT stability and the dimorphic switch require intracellular IP$_{8}$, which are down-regulated by Asp1 pyrophosphatase activity.
Fig 4. IP<sub>2</sub> controls Cut7-GFP spindle association.

A: Photomicrographs of cut7<sup>-gfp</sup> cells transformed with a vector control or an asp<sub>1</sub><sup>365-920</sup> expressing plasmid. Scale bar= 2 μm. B: Quantification of the fluorescence signal of Cut7-GFP on short spindles. For relative signal intensity at the spindle midzone compared to the spindle ends, the fluorescence signal at the midzone was normalized against the background (square 5 – square 6) and divided by the fluorescence intensity at spindle ends (square 1 – square 2 and square 3 – square 4). C: Diagrammatic representation of the ratios spindle midzone/spindle ends (control: n= 29; pasp<sub>1</sub><sup>365-920</sup> n= 24; ***: P ≤ 0.001, t-test; significant outliers removed using Grubbs' test.). D: Diagrammatic representation of the frequency of spindle breaks in the indicated transformants (control: n= 30; pasp<sub>1</sub><sup>365-920</sup> n= 29; ***: P ≤ 0.001, χ<sup>2</sup>-test). E: Diagrammatic representation of the ratios spindle midzone/spindle ends (control: n= 30; pasp<sub>1</sub><sup>365-920</sup> non-breaking n= 23; pasp<sub>1</sub><sup>365-920</sup> breaking n= 17 (9 cells); ***: P ≤ 0.001, *: P ≤ 0.05, t-test). F: Diagrammatic representation of the ratios spindle midzone/spindle ends (asp<sub>1</sub><sup>+</sup> cut7-GFP: n= 29; asp<sub>1</sub><sup>D333A</sup> cut7-GFP: n= 24; ***: P ≤ 0.001, t-test; significant outliers removed using Grubbs' test). Analysis was carried out at 33 °C.
Fig 5. *asp1*<sup>D333A</sup> cell population contains polyploid cells.

A: Photomicrographs of a mitotic *asp1*<sup>D333A</sup> *bub3Δ* cell expressing *sad1*<sup>+</sup>-mCherry and cen1-GFP. Time between images: 1 min. Scale bar= 2 µm. 2/11 analyzed *asp1*<sup>D333A</sup> *bub3Δ* double mutant cells showed this phenotype. B: FACS analysis of the indicated strains. Cells were gated for size revealing that cell populations with an *asp1*<sup>D333A</sup> background were much more heterogenous than *asp1*<sup>+</sup> populations. The P2 area contains the largest cells. C: Measurement of DNA content (2-32N) of the indicated cell population; left: entire population; right: P2 population. DNA content of peaks was defined by using the *cdc11-123* strain as standard (Suppl. Fig 5) (65).
Fig 6. Loss of functional Asp1 pyrophosphatase domain results in increased IP8 levels.

A: Diagrammatic representation of Asp1 variants analyzed. All variants were expressed from the endogenous asp1+ locus. B and C: HPLC elution profile of inositol polyphosphates of the wild-type type (WT) or asp1H397A strain. D: Comparison of part of the inositol pyrophosphate profiles of the wild-type and asp1H397A strains. E: Diagrammatic representation of IP8 levels relative to IP7. (WT: n= 4; asp1H397A n= 3; *: P ≤ 0.05, t-test). The fold-change of IP8/ IP7 is 2.81 higher for the asp1H397A strain compared to the wild-type strain. F: HPLC elution profile of inositol polyphosphates of the asp11-364 strain. G: Comparison of inositol pyrophosphate profiles of the wild-type and asp11-364 strains (data used for this wild-type were obtained from a strain grown in parallel to the asp11-364 strain) and H: Diagrammatic representation of IP8 levels relative to IP7 and normalized to the wild-type. (WT: n= 4; Asp11-364 n= 3; **: P ≤ 0.01, t-test). The fold-change of IP8/ IP7 is 1.67 higher for the asp11-364 strain compared to the wild-type strain.
Fig 7. The conserved amino acids of the M1 motif are essential for pyrophosphatase activity.

A: Diagrammatic representation of Asp1 pyrophosphatase M1 motif mutants. B: *In vitro* pyrophosphatase assay with using Asp1<sup>365-920</sup>, Asp1<sup>365-920/H397A</sup>, Asp1<sup>365-920/R400A</sup> or Asp1<sup>365-920/R396A</sup>. 8 µg of the indicated proteins were added to Asp1 kinase generated IP<sub>7</sub> (input shown in lane 1), incubated for 16 h and the resulting inositol polyphosphates resolved on a 35.5% PAGE and stained with Toluidine Blue; - component not added, + component added. All pyrophosphatase variants were tested at least twice in the *in vitro* assay. C: Serial dilution patch tests (10<sup>4</sup> to 10<sup>1</sup> cells) of a wild-type strain transformed with vector (control) or plasmids expressing the indicated *asp1* variants via the *nmt1*<sup>+</sup> promoter. Transformants were grown under plasmid selective conditions with or without thiamine and with or without 7 µg/ml TBZ at 25°C for 7 days. D: Serial dilution patch tests (10<sup>4</sup> to 10<sup>1</sup> cells) of an *asp1Δ* strain transformed with vector (control) or plasmids expressing *asp1*</sup> or *asp1*R<sup>400A</sup> from the *nmt1*<sup>+</sup> promoter. Transformants were grown as in C. E: Invasive growth assay. Left: 10<sup>5</sup> wild-type cells transformed with vector control, *asp1*</sup>, *asp1*</sup>H<sup>397A</sup> or *asp1*</sup>R<sup>400A</sup> plasmids were grown on plasmid selective thiamine-plus medium for 21 days at 30°C (top panels, surface growth). Removal of surface growth by washing revealed invasively growing colonies (bottom panels). F: Quantification of invasively growing colonies; per plasmid 3 transformants were analyzed in triplicate, ***P < 0.0005, t-test. Number of invasive colonies: 81 ± 6 for *asp1*</sup>H<sup>397A</sup> and 113 ± 8 for *asp1*</sup>R<sup>400A</sup>. 
Fig 8. The I808D mutation abolishes Asp1 pyrophosphatase activity

A: Diagrammatic representation of M2 motif mutants. B: \textit{In vitro} pyrophosphatase assay using 8 µg protein of the indicated Asp1 variants. Assay performed as in Fig. 7B. The pyrophosphatase variants were tested at least twice in the \textit{in vitro} assay. C: Serial dilution patch tests (10⁴ to 10¹ cells) of a wild-type strain transformed with vector (control) or plasmids expressing \textit{asp1}^{365-920} or \textit{asp1}^{365-920/I808D} via \textit{nmt}1⁺. Transformants were grown under plasmid selective conditions with or without thiamine and with or without 7 µg/ ml TBZ at 25°C for 7 days. D: Serial dilution patch tests (10⁴ to 10¹ cells) of wild-type, asp1Δ, \textit{asp1}^{H397A} and \textit{asp1}^{I808D} strains grown on YE5S full media at 25°C for 5 days with or without 12 µg/ml TBZ. E: HPLC elution profile of inositol polyphosphates of the \textit{asp1}^{I808D} strain. F: Comparison of inositol pyrophosphate profiles of wild-type and \textit{asp1}^{I808D} strains. G: IP₈ levels relative to IP₇ and normalized to the wild-type from data shown in F. (WT: n= 4; Asp1^{I808D} n= 3; *: P ≤ 0.05, t-test). The fold-change of IP₈/ IP₇ is 2 for the \textit{asp1}^{I808D} strain compared to the wild-type strain.
### Table A

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### Image B

- YE55S: ![Image](image11.png)
- MM: ![Image](image12.png)
- MM+Met: ![Image](image13.png)
- MM+Cys: ![Image](image14.png)
- MM+Cys+Met: ![Image](image15.png)

### Image C

- +thiamine (low expression): ![Image](image16.png)
- +thiamine (high expression): ![Image](image17.png)

### Image D

- -thiamine (low expression): ![Image](image18.png)
- -thiamine (high expression): ![Image](image19.png)

### Image E

- Coomassie staining: ![Image](image20.png)
- Far western blot analysis: ![Image](image21.png)
Fig 9. Characterization of the Asp1 interaction partner Met10.

A: Yeast-two-hybrid analysis of the interaction between Asp1 and Met10 (SPCC584.01c). S. cerevisiae strain AH109 was co-transformed with a plasmid expressing asp1+ fused to the GAL4 binding domain (pGBK7) and a plasmid expressing met10 variant (aa 544-1006) fused to the GAL4 activation domain (pGAD77). Cells were spotted on plasmid selective SD medium with or without histidine and incubated for 5 days at 30°C. B: Growth of wild-type and met10Δ strains on the indicated media: full media (YE5S), minimal media (MM), MM plus 330 µM cysteine (MM+Cys), MM plus 140 µM methionine (MM+Met) or MM plus cysteine and methionine (MM+Cys+Met). C: Serial dilution patch tests (10^4 to 10^1 cells) of a wild-type strain transformed with vector (control) or plasmids expressing asp1+ or met10+ from the nmt1+ promoter. Transformants were grown at 25°C for 8 days. D: Serial dilution patch tests (10^4 to 10^1 cells) of transformed asp1Δ cells with vector (control) or plasmids expressing asp1+ or met10+ via nmt1+. Incubation at 25°C for 11 days. E: Far western analysis. Far left: Coomassie stained gel of 1 µg of the indicated purified proteins. Left: Protein-protein interaction of GST-Met10 (blotted protein; 138 kDa, arrow) and Asp1^{365-920}-His (probe protein). Detection of GST-Met10 via His antibody. Middle: Control; Asp1^{365-920}-His (blotted protein) and GST (probe protein) using a GST antibody. Right: Protein-protein interaction of Asp1^{365-920}-His (blotted protein; 65 kDa, arrow) and GST-Met10 (probe protein). Detection of Asp1^{365-920}-His via GST antibody. 1 µg of protein was loaded on the gel in all cases. Concentration of probe proteins: 10 µg/ ml.
**A**

- MetU-GFP
- Mito tracker
- Merge

**B**

- asp1*
- asp1<sup>1597A</sup>
- asp1.1

**Mitochondrial distribution**

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- Black: tubular/wild-type-like
- Gray: aggregated/aberrant

**C**

*in vitro* pyrophosphatase assay

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- GST: Glutathione S-transferase
- Ddp1: Deoxypyrimidine deaminase 1
- Met10: Methionine adenosyltransferase 10
- Asp<sup>1597A</sup>: Aspartate
- GST-Asp<sup>1597A</sup>-His: GST fusions
- GST-Asp1<sup>1597A</sup>-920: GST fusions
- Input: Input sample

![Western Blot Image]

- L: IP<sub>7</sub>
- I: IP<sub>6</sub>
- A: ATP
Fig 10. The mitochondrial associated Met10 protein inhibits Asp1 pyrophosphatase activity in vitro.

A: Live cell imaging of Met10-GFP cells stained with Mitotracker. Shown are maximum-intensity-projection images of interphase cells grown at 25°C. Bar, 10 μm.

B: Top: Live cell imaging of the mitochondrial protein Cox4-RFP in asp1Δ or asp1Δ cells. Shown are maximum-intensity-projection images of interphase cells grown at 25°C. # normal and * abnormal mitochondrial distribution. Bar, 10 μm. Bottom: quantification of mitochondrial distribution: asp1Δ strain, n = 44; asp1Δh397A strain, n = 77; asp1Δ strain, n=44; P** < 0.01, P*** < 0.001 χ² test). C: In vitro pyrophosphatase assay: input controls (lanes 1 and 8), 4 μg GST-Asp1365-920 (lane 2), 4 μg GST-Asp1365-920 plus 6 μg Met10 (lane 3). In vitro pyrophosphatase assay using 4 μg Asp365-920-His (lane 4) or 4 μg Asp365-920-His plus 8 μg Met10 (lane 5). In vitro pyrophosphatase assay using 2 μg Ddp1-GST (lane 6) or 2 μg Ddp1-GST plus 6 μg Met10 (lane 7). Lane 9 shows addition of 2 μg GST. In vitro pyrophosphatase assay using 6 μg GST-Met10 and 2 μg GST (lane 10). In vitro pyrophosphatase assays involving Met10 protein were repeated 4 times. All assays were incubated for 16 h and the resulting inositol polyphosphates resolved on a 35.5% PAGE and stained with Toluidine Blue; - component not added, + component added. Size of proteins used in C: GST-Asp1: ~91 kDa, GST-Met10: ~138 kDa, Asp1365-920-His: ~66 kDa, GST-Ddp1: ~48 kDa.