Phospholipase C of Cryptococcus neoformans regulates homeostasis and virulence by providing inositol trisphosphate as a substrate for Arg1 kinase

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Running title: Arg1 function in C. neoformans

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Phospholipase C of Cryptococcus neoformans (CnPlc1) is crucial for virulence of this fungal pathogen. To investigate the mechanism of CnPlc1-mediated signaling, we established that phosphatidylinositol 4,5-bisphosphate (PIP2) is a major CnPlc1 substrate, which is hydrolyzed to produce inositol trisphosphate (IP3). In Saccharomyces cerevisiae, Plc1-derived IP3 is a substrate for the inositol polyphosphate kinase Arg82, which converts IP3 to more complex inositol polyphosphates. We show that in C. neoformans, the enzyme encoded by ARG1 is the major IP3 kinase, and we further demonstrate that catalytic activity of Arg1 is essential for cellular homeostasis and virulence in the Galleria mellonella infection model. IP3 content was reduced in CnΔplc1 and markedly increased in CnΔarg1, while PIP2 was increased in both mutants. CnΔplc1 and CnΔarg1 shared significant phenotypic similarity, including impaired thermotolerance, compromised cell walls, reduced capsule production and melanization, defective cell separation and inability to form mating filaments. In contrast to ScΔarg82, CnΔarg1 exhibited dramatically enlarged vacuoles indicative of excessive vacuolar fusion. In mammalian cells, PLC-derived IP3 causes Ca2+ release and calcineurin activation. Our data show that, unlike mammalian PLCs, CnPlc1 does not contribute significantly to calcineurin activation. Collectively, our findings provide the first evidence that the inositol polyphosphate anabolic pathway is essential for virulence of C. neoformans, and further show that production of IP3 as a precursor for synthesis of more complex inositol polyphosphates is the key biochemical function of CnPlc1.
Introduction

_Cryptococcus neoformans_ var._ grubii_ is a human fungal pathogen that predominantly infects immunocompromised hosts. After inhalation from the environment, infection spreads from the lung to the brain via the blood stream, causing life-threatening meningo-encephalitis. Several factors contribute to the virulence of _C. neoformans_, including the ability to grow at physiological temperature (37°C) and to produce melanin and capsule (1-3). Our previous work demonstrated that these virulence determinants are attenuated by deletion of a single gene encoding a key signaling molecule, phosphatidylinositol (PI)-specific phospholipase C (Plc1). In addition, the _PLC1_ deletion mutant (CnΔplc1) had a defective cell wall and compromised virulence in both mouse (37°C) and _Caenorhabditis elegans_ (25°C) models of cryptococcosis (4). A second PLC homolog, _PLC2_, appears to have no significant role in the biology and virulence of _C. neoformans_ (4).

In mammalian cells, PI-specific phospholipase C enzymes (PLCs) catalyze the hydrolysis of PI-(4,5)-bisphosphate (PIP2) to diacylglycerol (DAG) and D-myo-inositol-(1,4,5)-trisphosphate (IP3). DAG directly activates protein kinase C (PKC), while IP3 triggers release of calcium from intracellular stores via interaction with IP3 receptors in the ER. Elevated cytosolic calcium activates calcium-dependent signaling enzymes, such as calcineurin. In the model yeast _S. cerevisiae_, a single PLC isoform has similar catalytic activity to the mammalian PLCδ isoform (5). However, the role of the PIP2 breakdown products and the mechanism of Plc1-dependent signaling remain to be fully elucidated. Similarly, in _C. neoformans_, the identity and fate of the CnPlc1 hydrolysis products has not been determined.
In fungi, the role of PIP2-derived IP3 in initiating calcium influx is controversial as no homologs of mammalian IP3 receptors have been identified in fungal genomes. In the filamentous fungus *Neurospora crassa*, the existence of a functional IP3 receptor equivalent has been inferred on the basis of an IP3-dependent increase in cytoplasmic Ca^{2+}. *N. crassa* PLC is thought to be activated by membrane stretch, generating IP3 which triggers Ca^{2+} release from hyphal tip vesicles, thus maintaining a high Ca^{2+} concentration required for hyphal tip growth (6). In *S. cerevisiae*, however, transient IP3 increase in nitrogen-depleted cells following addition of ammonium sulphate did not trigger a spike in intracellular calcium (7). In glucose-starved *S. cerevisiae*, glucose addition caused PLC-dependent influx of extracellular calcium accompanied by a transient increase in IP3. However, it is unclear as to whether calcium influx is triggered by IP3, or via direct interaction of PLC with a plasma membrane calcium channel (8, 9).

In fungi, increased cytosolic calcium triggered by stress or morphological changes, activates the protein phosphatase, calcineurin, via calcium-bound calmodulin. In *C. neoformans*, calcineurin responds to stress caused by cell wall perturbing agents, host physiological temperature, high CO2, alkaline pH and high cation concentration. Moreover, calcineurin is essential for virulence and hyphal elongation during mating and monokaryotic fruiting (10, 11).

In *S. cerevisiae*, calcineurin conducts most of its transcriptional regulation via activation of the zinc finger transcription factor, Crz1. In *C. neoformans*, the Crz1 ortholog is also activated by calcineurin and, during starvation, by protein kinase C, to regulate cell wall integrity (12, 13).

An alternative fate of PLC-generated IP3 in *S. cerevisiae* is phosphorylation by inositol polyphosphate kinases (IPKs). The resulting inositol polyphosphates (IPs) influence chromatin remodeling, transcriptional regulation, mRNA nuclear export, telomere length, vacuole morphogenesis, endocytosis and cell division via an effect on kinetochore activity (14, for
In *S. cerevisiae* the single IPK, Arg82, converts Plc1-derived IP3 into IP4 and IP5, which can be further phosphorylated to IP6 by Ipkl. Kcs1 and Vip1 then convert IP6 to different isoforms of inositol pyrophosphate (PP-IP), PP-IP5. PP-IPs contain two phosphates on the same position of the inositol ring. Arg82 and Kcs1 can also use IP5 as a substrate to generate two isoforms of PP-IP4 (16). Similar to deletion of *PLC1* in *C. neoformans*, deletion of *ARG82* in *S. cerevisiae* results in a pleiotropic phenotype. ScΔarg82 abnormalities include temperature sensitivity, sterility and defective sporulation. We therefore hypothesized that the key role of Plc1 in *C. neoformans* is to produce IP3 as a precursor for the synthesis of more complex IPs, and that the deletion of the ARG82 ortholog in *C. neoformans* would produce a pleiotropic phenotype similar to that of CnΔplc1 due to the lack of IP4-8 species in both mutants.

In this study we investigated the biochemical mechanism underlying Plc1-mediated signaling in *C. neoformans*. Our findings suggest that the main function of cryptococcal Plc1 is to supply IP3 as a substrate for an inositol polyphosphate kinase, Arg1, and that the catalytic activity of Arg1 is essential for cellular homeostasis and virulence of *C. neoformans*.

### Materials and Methods

#### Strains and media

Wild-type *C. neoformans* var. *grubii* strain H99 (serotype A, MATa) was used in this study. The Δcna1 mutant and the KN99 (MATα) strain were a kind gift from Joseph Heitman’s laboratory (Duke University, NC, USA). The Δmpk1 mutant was also a gift from Jenny Lodge’s laboratory (Washington University in St Louis USA). *C. neoformans* strains were routinely cultured on
YPD medium (1% yeast extract, 2% peptone and 2% dextrose). Mating was induced on 5% V8 juice (pH 5), 2% agar plates. Minimal Medium (MM, 15mM glucose, 10mM MgSO₄, 29.4mM KH₂PO₄, 13mM glycine, 3µM thiamine) was used for capsule induction. MM agar plates supplemented with 1 mM L-DOPA were used to assess melanization.

**CnPlc1 expression in S. cerevisiae**

The *C. neoformans* PLC1 (CNAG_02867) coding sequence (see Table 1 for primer sequences) was amplified from a *C. neoformans* cDNA library (a gift from Peter Williamson, NIH), cloned into the pYES2/NT vector (Life Technologies) and transformed into *S. cerevisiae* strain INVSc1. Expression of *CnPLC1* was induced by growing the cells in Synthetic Defined medium (SD) without uracil, replacing glucose with galactose as the sole carbon source. For Plc1 protein purification, yeast cells were processed as previously described (5). In brief, the cells were lysed in Buffer A [50 mM Tris (pH 7.9), 400 mM NaCl, 1 mM PMSF and protease inhibitor cocktail] by vigorous vortexing with 0.5 mm glass beads. The protein extracts were clarified by centrifugation and subjected to Ni²⁺-affinity chromatography. The eluted fractions that were demonstrated to contain Plc1 protein were combined and dialyzed against Buffer B [25 mM HEPES-HCl (pH 7.2), 50 mM NaCl, 1 mM EDTA]).

**PLC activity assay**

The PLC-specific activity of purified CnPlc1 and the crude protein extracts was determined using a radiometric enzyme assay, with PI or PIP₂ as the substrate as described previously (5) with modifications. For the PIP₂ hydrolysis assay, the substrate was prepared by combining 10 nmol of cold L-α-phosphatidylinositol 4,5-diphosphate (Sigma, P9763) dissolved in 20 µl
chloroform:methanol:water:1N HCl 20:10:1:1 (v/v/v/v)) with 1.5 µl (0.015 µCi) of hot phosphatidylinositol-4,5-biphosphate [inositol-2-³H(N)] (Perkin-Elmer, NET895005UC) per reaction. PIP₂ substrate mix was dried under nitrogen and resuspended in 25 µl of 2X PIPLC-PIP₂ Assay Buffer [PPAB; 1.6 mM Triton X-100, 100 mM Hapes (pH 7.0), 200 mM NaCl, 2 mM EGTA, 2 mM CaCl₂] per reaction. Substrate dissolution was aided by sonicating in a water bath sonicator (Soniclean, SA, Australia) for 10 min with occasional vortexing. Each assay was initiated by the addition of 25 µl of substrate/PPAB to 5-25 µg protein. The final volume of the reaction was adjusted to 50 µl. Incubation was carried out at 30°C for 15 min. The tubes were placed on ice and 100 µl of 1% BSA was added to each tube followed by 250 µl of 10% TCA. The tubes were centrifuged and the ³H radioactivity in the supernatants (350 µl) was determined by scintillation counting.

To determine the PIPLC activity using PI substrate, the assay was conducted at 37°C in a final volume of 100 µl. For each reaction, 25 nmol of cold L-α-phosphatidylinositol (PI) (Sigma, P8443) dissolved in chloroform and 0.125 µl (0.025 µCi) of phosphatidylinositol, L-α-[myo-inositol-2-³H(N)] (Perkin-Elmer, NET862010UC) were dried under nitrogen and resuspended in 50 µl of 2X PIPLC-PI Assay Buffer [PIAB; 0.2% sodium deoxycholate, 100 mM Hapes (pH 7.0), 200 mM NaCl, 4 mM EGTA, 0.1% BSA]. Substrate dissolution was aided by sonicating in a water bath sonicator (Soniclean, SA, Australia) for 10 min with occasional vortexing. The assay was initiated by adding 50 µl of substrate/PIAB to 10-25 µg protein in a final reaction volume of 100 µl. The reactions were incubated at 37°C for 10 min, and terminated by adding 0.5 ml chloroform:methanol:HCl (100:100:0.6, v/v/v), followed by 0.15 ml of 1 M HCl containing 5 mM EGTA, and vigorous vortexing. The reaction tubes were centrifuged at 21,000g
for 1 min to achieve phase separation. \(^3\)H radioactivity in the aqueous phase (250 \(\mu\)l) was determined by scintillation counting.

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\text{PIP}_2 \text{ hydrolysis was quantified as follows: } \mu\text{moles PIP}_2 \text{ hydrolysed/minute/mg of protein} = \frac{[\text{released cpm} - \text{blank cpm}]}{\text{total cpm}} \times 0.01 \mu\text{moles PIP}_2 / (\text{mg of protein} \times 15 \text{ min}).
\]

Similarly, PI hydrolysis was quantified as follows: \(\mu\)moles PI hydrolysed/minute/mg of protein = \(\frac{[\text{released cpm} - \text{blank cpm}]}{\text{total cpm}} \times 0.025 \mu\text{moles PI} / (\text{mg of protein} \times 10 \text{ min}).\) “Blank” refers to a reaction without protein, which was included in each assay to determine the level of spontaneous substrate breakdown.

**IP3 and PIP2 quantification**

*C. neoformans* cultures grown overnight in YPD broth were pelleted, resuspended in water to an OD\(_{600}\)=10, spotted on YPD plates and incubated overnight at 30\(^\circ\)C. 40-80 mg of Cryptococcal cells were scraped from the plates, weighed, quenched by adding 4 ml of ice-cold (<-20\(^\circ\)C) 60% methanol and vortexed vigorously (7, 9). Quenched cells were pelleted by centrifugation and resuspended in ice-cold 4% perchloric acid (1 ml per 75 mg cells). To release IP3, glass beads were added to the tubes, and the cells were homogenized in a bead beater (4 x 30 sec cycles of beating at 4\(^\circ\)C, at 1 min intervals). Soluble and insoluble fractions were separated by centrifugation (5 min at 10,000 rpm, 4\(^\circ\)C) to quantify IP3 and PIP2, respectively.

The pH of the IP3-containing supernatant was adjusted to 7.5 by titrating with ice-cold 1.5M KOH, 60mM HEPES. pH was monitored during the titration using a universal pH indicator strip. The KClO\(_4\) formed during the titration was sedimented by centrifugation at 2,000g for 15 minutes at 4\(^\circ\)C. The Inositol-1,4,5-Trisphosphate \([\ ^3\text{H}\] Radioreceptor Assay Kit (PerkinElmer...
Life Sciences) was then used to measure IP$_3$ concentration in the supernatant (100μl/reaction) following the manufacturer’s instructions.

To quantify PIP$_2$, the insoluble pellet obtained by centrifugation of the homogenized cells was washed with 1.5 ml of ice-cold water, resuspended in 940 μl chloroform:methanol:HCl (80:40:1 v/v) and vortexed for 15 min. Lipid and aqueous phases were resolved by adding 310 μl chloroform and 560 μl 0.1M HCl, vortexing and centrifuging at 1,000g for 15 minutes at room temperature. 400 μl of the lower phase was dried under a stream of nitrogen. The dried samples were dissolved in 250 μl of 1 M KOH and heated at 100°C for 15 min to hydrolyze PIP$_2$. After cooling on ice, the samples were supplemented with 15 μl HEPES 1 M, pH 7.2 and further neutralized to pH 7.5 by titrating with ice-cold 4% perchloric acid. The samples were centrifuged at 2,000g for 15 minutes at 4°C to remove KClO$_4$ sediment. The IP$_3$ produced as a result of PIP$_2$ hydrolysis (100 μl of supernatant/reaction) was quantified using the Inositol-1,4,5-Trisphosphate [3H] Radioreceptor Assay Kit (PerkinElmer Life Sciences).

**Generation of transgenic strains**

Plasmid pJAF (containing a Neomycin resistance cassette, NEO$^R$) was kindly provided by Dr John R Perfect, Duke University, Durham, NC, USA. The ARG1 and ARG2 gene deletion constructs were made by overlap PCR, joining the 5’ flank, NEO$^R$ from pJAF (ACT1 promoter, neomycin phosphotransferase, TRP1 terminator) and the 3’ flank (see Fig. S1-A for diagram and Table 1 for primer sequences). Transformation was carried out using the biolistic method (17). NEO resistant transformants were selected on YPD agar plates supplemented with 0.5 M sorbitol and 100 μg/ml G418. Correctly targeted integration of the ARG1 and ARG2 deletion constructs was confirmed by PCR amplification across the junction point of integration of the construct.
with genomic DNA, using a forward primer that anneals outside the region of integration and a reverse primer that anneals within the construct (Fig. S1). The \( \Delta \text{arg}1: \text{ARG1} \) reconstituted strain was created by transforming the \( \Delta \text{arg}1 \) mutant with a genomic fragment of \( \text{ARG1} \) which included 1,169 bp upstream and 1,133 bp downstream of the coding region (primers 5’s – 3’a). To select for \( \text{ARG1} \)-expressing colonies, transformed cells were plated on YPD agar supplemented with 0.5 M sorbitol and 0.005% Congo Red, and incubated at 37°C. To confirm that the colonies which grew under these conditions were true \( \Delta \text{arg}1: \text{ARG1} \) reconstituted strains, the cells were tested for the presence of both ectopically-integrated \( \text{ARG1} \) and the \( \text{arg}1 \) deletion cassette by PCR (Fig. S1).

**Virulence in Galleria mellonella**

*\( C. \text{neoformans} \)* WT and mutant strains were grown overnight, pelleted by centrifugation and resuspended in PBS at a concentration of \( 10^8 \) cells/ml. *\( G. \text{mellonella} \)* larvae (10 per strain) were inoculated with \( 10^6 \) yeast cells by injection into the hemocoel via the lower pro-legs. The viability of each inoculum was assessed by performing serial 10-fold dilutions, plating the lowest dilutions on SAB plates and counting the colony forming units after 3 days incubation at 30°C. Inoculated larvae were monitored daily for 10 days. The Kaplan-Meier method in the SPSS version 21 statistical software was used to estimate the differences in survival (log-rank test) and to plot the survival curves. In all cases a P-value <0.05 was considered statistically significant.

The number of viable fungal cells in larvae was determined by homogenizing each larva in 1ml PBS, and preparing and plating serial dilutions of each homogenate, as described for the inoculum.
Gene expression
To compare chitin synthase CHS6 (CNAG_00546) expression, the cells were cultured in YPD overnight at 30°C and diluted to OD 0.5. After 4 hours incubation, the cells were treated with calcofluor white (2.2 mg/ml) with or without FK506 (10 μg/ml) for 1 hour. The cells were collected and snap-frozen. RNA extraction and cDNA synthesis were performed as described (12). Real time PCR was performed using the actin-encoding gene (ACT1) for normalization.

Microscopy
For staining of C. neoformans vacuoles, the cells were grown in YPD overnight, diluted 1:10 in fresh medium and incubated for 4 hours. The cells were pelleted and resuspended in 50 mM sodium citrate buffer, pH 5, supplemented with 1 μM Carboxy-DCFDA (Life Technologies), and incubated for 20 min. Measurements of the vacuolar size were performed using ImageJ software (NIH). For the FM-4-64 pulse-chase experiment, the YPD-grown cells were loaded with 10 μM FM-4-64 in YPD for 5 minutes (time point 0), pelleted and resuspended in fresh YPD medium. The tubes were incubated at 30°C with shaking. Aliquots were withdrawn at the indicated times, and the cells pelleted and kept on ice until observed by microscopy.

For F-actin staining, the cells were grown overnight in YPD, pelleted and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were washed twice with PBS and permeabilized with 1% Triton X-100 in PBS for 8 min. Following permeabilization, the cells were washed twice with PBS, and incubated with 1% BSA in PBS for 20 min, followed by staining with Alexa Fluor 488-conjugated phalloidin in PBS (Life Technologies, 25U/100 μl
cells) for 90 min. DAPI (4',6-diamidino-2-phenylindole, 1 μg/ml) was added for the final 3 min of the 90 min incubation with Alexa Fluor 488-conjugated phalloidin.

Results

CnPlc1 preferentially hydrolyses PIP$_2$

To establish that Plc1 is enzymatically active and to determine its substrate preference, we expressed recombinant HIS-tagged CnPLC1 in S. cerevisiae under the regulation of a galactose-inducible promoter (Figure 1A). The enzyme was then purified by nickel affinity chromatography. Purified enzyme and crude cell lysates were assayed for PI-PLC hydrolytic activity using PI or PIP$_2$ as a substrate in the presence or absence of calcium (Figure 1B). No activity was obtained when calcium was omitted from the buffer (not shown). However in the presence of calcium, PIP$_2$, but not PI, was hydrolyzed by both pure and crude CnPlc1, confirming that PIP$_2$ is a preferred substrate of CnPlc1. These data supports our previous finding where the PIP$_2$ hydrolytic activity associated with Aplc1 lysates was significantly lower than the PI hydrolytic activity, when compared to the respective WT levels (4). Preference for PIP$_2$ indicates that CnPlc1 can produce both IP$_3$ and DAG, rather than DAG alone, as would occur if PI was the preferred substrate.

Identification of the major inositol polyphosphate kinase (Arg1) in C. neoformans

In S. cerevisiae PLC-derived IP$_3$ is converted to IP$_4$ and IP$_5$ by the inositol polyphosphate multikinase Arg82 (16, 18). Using the S. cerevisiae protein in a similarity search, we identified two Arg82 homologs in the C. neoformans genome: CnArg1 (CNAG_06500, NCBI accession
AFR98730) and CnArg2 (CNAG_02802, NCBI accession AFR93890), which share 22% and 15% identity, respectively, with ScArg82, and 17% identity with each other (see Fig. S2 for alignment). Similar to ScArg82, CnArg1 and CnArg2 contain conserved PDKG motif essential for the catalytic activity of IP3 kinases (19, 20).

To determine whether CnArg1 and CnArg2 are functional IP3 kinases, we created the ARG1 and ARG2 deletion mutants, CnΔarg1 and CnΔarg2, respectively. We then compared the IP3 content of CnΔarg1 and CnΔarg2 with that of WT and CnΔplc1. Due to the absence of functional CnPlc1, we expected the IP3 content of CnΔplc1 to be reduced. Due to the absence of functional IP3 kinase, we expected to see an accumulation of Plc1-derived IP3 in CnΔarg1 and/or CnΔarg2. Consistent with our predictions, IP3 content was reduced 3-fold in CnΔplc1 and markedly increased (>5-fold) in CnΔarg1 (Fig. 2A). In contrast to CnΔarg1, the IP3 content in CnΔarg2 was similar to WT (Fig. S3-A). To ascertain the authenticity of the CnΔarg1 phenotype, we created CnΔarg1:ARG1 reconstitution strain by transforming CnΔarg1 with a genomic ARG1 fragment (Fig. S1). The IP3 content in CnΔarg1:ARG1 was intermediate between WT and CnΔarg1, consistent with partial restoration of the Δarg1 phenotype by the introduction of ARG1. Accumulation of IP3 in CnΔarg1 establishes CnArg1 as the major IP3 kinase in C. neoformans.

In addition to reduced IP3 content, deletion of PLC1 potentially leads to accumulation of its PIP2 substrate, with a resultant deleterious effect on yeast cells (21). We therefore measured the PIP2 membrane content in Δplc1 and Δarg1 indirectly, by measuring IP3 produced by alkaline hydrolysis of PIP2 (Fig. 2B). Surprisingly, PIP2 accumulation was even more pronounced in Δarg1 than in Δplc1. This finding implies product inhibition of Plc1 activity in Δarg1 by the high concentration of IP3.
Arg1 is essential for virulence, cell wall integrity and mating in C. neoformans

Changes in IP$_3$ content in $\Delta$plc1 and $\Delta$arg1 (Fig. 2A) demonstrate that Plc1 and Arg1 function within the same signaling pathway, with Plc1 providing a substrate (IP$_3$) for Arg1. Arg1 catalytic products and their derivatives are expected to be absent in both mutants, as is the case in S. cerevisiae (22, 23). To assess the role of Arg1 and its products in virulence and homeostasis, we characterized the $\Delta$arg1 phenotype, as compared to $\Delta$plc1 and the wild type strain.

Similar to $\Delta$plc1, growth of $\Delta$arg1 was delayed under normal growth conditions (30°C, YPD). However, growth of both strains was markedly inhibited at human physiological temperature (37°C) and in presence of the cell wall perturbing agent, CongoRed (Fig. 3A). Melanization on minimal medium containing L-DOPA (Fig. 3B), and the formation of mating filaments in a unilateral cross with KN99 (MATa, Fig. 3C), were also reduced. In the case of temperature stress and mating, the phenotype of $\Delta$arg1:ARG1 was completely restored to that of WT. However, in the case of Congo red sensitivity and melanization, the phenotype of $\Delta$arg1:ARG1 was intermediate between WT and $\Delta$arg1, consistent with partial restoration of the $\Delta$arg1 phenotype by the ectopically integrated ARG1. Both, $\Delta$plc1 and $\Delta$arg1 formed smaller capsules than the WT strain under capsule-inducing conditions, with capsule size restored to that of WT in $\Delta$arg1:ARG1 (Fig. 3D). Unlike $\Delta$arg1, $\Delta$arg2 exhibited WT-like tolerance to elevated temperature and cell wall stress (Fig. S3-B). $\Delta$arg2 also produced melanin and capsules similar to the WT strain (Fig. S3-C,D). The lack of phenotypic defects in $\Delta$arg2 is consistent with unaltered IP$_3$ content in this mutant (Fig. S3-A).

The virulence of $\Delta$arg1 and $\Delta$plc1 was compared with that of WT in larvae of the greater wax moth, Galleria mellonella. The G. mellonella infection model was chosen over the mouse inhalational model as larvae can be maintained at the permissive growth temperature of the
mutant strains (30°C). At mouse body temperature (~37°C), the growth of both mutants was severely compromised (Fig. 3A). Larvae were inoculated with 1 x 10⁶ cryptococci. The inoculum was plated out to quantify the inoculum as colony forming units (CFUs). This was determined to be 0.8, 0.5 and 1.1 x 10⁶ CFUs for WT, Δplc1 and Δarg1, respectively. While all of the larvae inoculated with WT *C. neoformans* died within 3 days, Δarg1-inoculated insects died more slowly (median survival of ~5 days) (Fig. 4). Unlike Δarg1, infection with Δplc1 caused the death of only one larva out of 10 over the entire infection period. Survival of the larvae was analyzed using the Kaplan-Meier method (log rank test). Overall, the difference in survival was statistically significant (p<0.001). For the pairwise comparisons, survival of PBS and Δplc1-infected larvae was comparable (p=0.317). Survival of PBS and Δplc1-infected larvae was significantly greater than that of Δarg1-infected larvae (p=0.001), which in turn was significantly greater than WT (p<0.001).

Fungal cells were retrieved from infected insects and quantified. WT cells had propagated dramatically within larvae that had succumbed to infection on day 3 post-inoculation, with ~128 fold more CFUs recovered compared with the number inoculated. Δarg1 also propagated within larvae, but not to the same extent as WT, with ~25 fold more CFUs recovered from larvae at death on day 4 post-inoculation. Interestingly, Δplc1 did not propagate in larvae, as similar CFUs to those inoculated were recovered from healthy larvae on day 6 post-inoculation.

**Altered vacuolar morphology, cell separation and endocytosis in Δplc1 and Δarg1**

We previously demonstrated that Δplc1 mutant cells had irregular morphology and contained enlarged vacuoles, as compared with WT (4). Similar to Δplc1, Δarg1 cells appeared to have enlarged vacuoles (Fig. 3D). To further investigate vacuolar morphology, vacuolar lumens were...
stained with carboxy-DCFDA and vacuole size and number per cell was documented (Fig. 5A,B). Cells of all strains contained numerous small vacuoles. However, Δarg1 cells often contained one markedly enlarged vacuole, implying excessive vacuolar fusion in this mutant (Fig 5A, arrowheads). The average vacuole size in Δarg1 (2.2μm) is 3-fold larger than that of WT (0.63μm), while the vacuoles of Δplc1 (average size 0.97μm) are only slightly larger than the vacuoles of WT (Fig. 5B). In all strains, the number of vacuoles per cell inversely correlated with vacuole size. On average, WT cells contained 3.5 vacuoles, as compared to 1.3 and 2 vacuoles in Δarg1 and Δplc1, respectively (Fig. 5B).

Δplc1 and Δarg1 cells often formed aggregates of three or four conjoined cells (Figure 5C, broken arrows), while WT cells were only joined in pairs. Since defects in morphology and cell separation might reflect abnormal polarized growth, WT, Δplc1 and Δarg1 were stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin distribution. In all strains, actin patches were distributed throughout the cytosol of mature cells and accumulated in the emerging buds, as described previously (24). In WT, most of the conjoined cells were pre-mitotic, with nuclei visible only in the mother cell. However, in Δplc1 and Δarg1, cells often failed to separate after mitosis, and nuclei were visible in both mother and daughter cells. Furthermore, brightly stained actomyosin rings were often observed at the junctions between these conjoined mutant cells (Fig 5C, solid arrows). These actomyosin rings eventually disappeared, and the actin patches became re-distributed throughout the conjoined cells. Despite being joined, these cells sometimes formed new buds. Actomyosin rings were rarely observed in WT cells. A similar cell separation defect accompanied by increased localization of F-actin in the medial ring was observed in *Schizosaccharomyces pombe* deficient in the IP5 2-kinase, Ipkl (25).
In *S. cerevisiae*, inositol pyrophosphates regulate endocytic trafficking (23). As we expected inositol pyrophosphates to be absent from CnΔ*plc1* and CnΔ*arg1*, we investigated the dynamics of endocytosis in these mutants by tracking internalization of the lipophilic dye, FM4-64 (Fig. 6). The cells were labeled with the dye for 5 minutes, and then chased with fresh medium for different time periods to follow the progress of endocytosis. After 10 minutes chase, FM4-64 staining in WT was visible in small vesicles and endosomal and, possibly, vacuolar membranes, while in Δ*arg1*, FM4-64 had reached the membranes of large vacuoles, a hallmark of this mutant (Fig. 5). In most Δ*plc1* cells, FM4-64 was still associated with the plasma membrane after 10 minutes chase. Between 10 and 60 minutes, the FM4-64 staining pattern of WT and Δ*arg1* cells remained largely unchanged. In Δ*plc1*, the dye became visible inside the cells after 40 minutes chase, and by 60 minutes, had stained endosomal and vacuolar membranes (Fig. 6).

**CnPlc1 does not contribute significantly to calcineurin activation**

In mammalian cells, *PLC1* is a well-established regulator of IP$_3$-mediated calcium release into the cytosol, and cytosolic calcium activates calcineurin. In *C. neoformans*, Δ*plc1* shares several phenotypic characteristics with the calcineurin deletion mutant, Δ*cna1*: enlarged cells at 30°C, sensitivity to Congo Red, growth retardation at 37°C and a mating defect (Fig. 3) (4, 10, 12). However, there are also significant phenotypic differences between these mutants: only Δ*cna1* is sensitive to CFW (see below), and has enlarged capsules (our unpublished observation).

To determine whether Plc1 is essential for calcineurin activation, we investigated whether the Δ*plc1* defects, which are common to Δ*cna1*, could be rescued by supplying exogenous calcium to the Δ*plc1* growth medium. Our previous findings established that, similar to other fungal species, *C. neoformans* calcineurin is activated by the addition of exogenous calcium to the growth
medium (12). Furthermore, in filamentous fungi, addition of calcium restored some of the defects observed in Δplc1, such as growth rate in Cryphonectria parasitica, and formation of infection structures (appressoria) in Magnaporthe oryzae (26, 27). In S. cerevisiae, addition of calcium to the medium allowed Δplc1 to grow at 38°C (28). However, addition of calcium to C. neoformans Δplc1 (50 mM or 5 mM CaCl₂) did not restore mating ability, growth at 37°C or resistance to cell wall stress, indicating that impaired calcineurin activation in the absence of Plc1 is not the cause of the defects observed in this mutant (Fig. 7).

We previously demonstrated that the expression of the chitin synthase-encoding gene, CHS6, is up-regulated in response to cell wall perturbation with CFW, and that CHS6 expression requires functional calcineurin (12). To assess the activity of calcineurin in the absence of Plc1, we tested CHS6 expression in Δplc1, as compared to WT and Δcna1. CHS6 expression was reduced ~2-fold in Δplc1, as compared to WT. However, upon addition of the calcineurin inhibitor, FK506, CHS6 expression dropped by ~12 and 25 fold in WT and Δplc1, respectively. The reduction of CHS6 expression in Δplc1 indicates that calcineurin is active in this mutant (Fig. 8A).

Furthermore, growth of Δplc1 was inhibited in the presence of FK506 at 30°C, while Δcna1 grew normally, indicating that calcineurin is essential at this growth temperature in the absence of Plc1 (Fig. 8B). Moreover, calcineurin inhibition caused Δplc1 to become more sensitive to CFW than Δcna1. In C. neoformans, protein kinase C regulates cell wall integrity via activation of MAP kinase cascade, in which Mpk1 is a terminal kinase (29, 30). This pathway functions independently of calcineurin, although cross-talk between the two pathways has been observed (30, 31). Similar to Δplc1, growth of the cell wall integrity pathway mutants, Δpkc1 and Δmpk1, was inhibited by FK506 in the presence and absence of CFW. These findings suggest that Plc1,
calcineurin and the Pkc1/Mpk1 cell wall integrity pathway function in parallel to maintain cell wall integrity. Although we cannot rule out the possibility of cross-talk between the Plc1 and calcineurin pathways, collectively our results suggest it is unlikely that Plc1 contributes significantly to calcineurin activation in *C. neoformans*.

**Discussion**

In this report we present for the first time evidence that metabolism of inositol polyphosphates is essential for the phenotypic expression of virulence determinants in human fungal pathogens. We have studied this pathway in the basidiomycete, *C. neoformans*, and show that the main biochemical and cellular role of Plc1 is to produce IP$_3$ as a substrate for the inositol polyphosphate kinase, Arg1. We have shown that Plc1 and Arg1, and therefore the inositol polyphosphate anabolic pathway, are key regulators of several important virulence attributes of *C. neoformans*, including cell wall integrity, growth at elevated temperature, melanin production and capsule biosynthesis, suggesting a broad role for inositol polyphosphates in cellular homeostasis, growth and pathogenicity of *C. neoformans*. Two further important findings from our study are that, unlike in mammalian cells, CnPlc1-derived IP$_3$ does not contribute significantly to calcineurin activation and that IP$_3$ homeostasis in *C. neoformans* regulates cellular function differently to the non-pathogenic model yeast, *S. cerevisiae*.

We have demonstrated that CnPlc1 has similar catalytic activity to the mammalian PLC δ-isoform since it preferentially hydrolyzes PIP$_2$ over PI in a Ca$^{2+}$ dependent manner, to produce IP$_3$ and DAG. CnPlc1 activity is consistent with the activity and substrate preference of other eukaryotic PLC enzymes, and the fact that hallmark domains of eukaryotic PLC proteins, the X
and a Y catalytic domain and a C2 calcium binding motif, are present in CnPlc1 (32). In mammalian cells the C2 motif is involved in calcium-dependent binding of PLC to membrane phospholipids and orientation of the catalytic domain toward the membrane surface (33). The ability of CnPlc1 to bind to the plasma membrane via its C2 domain would enable Plc1 to sense and respond to cell surface perturbations arising due to changes in the external environment. C. neoformans also has a second PLC, Plc2, which has a catalytic domain similar to bacterial PLCs, suggesting that functional similarity to prokaryotic enzymes. However, deletion of PLC2 produced no changes in cellular function or virulence (4).

We identified two putative inositol polyphosphate multikinases in C. neoformans genome, designated Arg1 and Arg2, with Arg1 being the closest homolog of ScArg82. Accumulation of IP3 in Δarg1, but not in Δarg2, established Arg1 as the major IP3 kinase in C. neoformans. This observation coupled with the fact that IP3 content is reduced in Δplc1, confirms that there is a block within the IP-processing pathway in both mutants. These data also demonstrate that Plc1 and Arg1 function within the same signaling pathway, with Plc1 providing a substrate (IP3) for Arg1.

While accumulation of the Plc1 substrate, PIP2, was expected in Δplc1, even higher PIP2 levels were detected in Δarg1. A similar phenomenon was observed in ScΔarg82 (23). It is possible that the excessive IP3 in CnΔarg1 competes with PIP2 for binding to the C2 domain of Plc1 and thus inhibits Plc1 enzyme activity. This hypothesis is supported by the finding that C2 domain-containing PLC of a squid binds IP3 (34). A functionally similar phenomenon of feedback inhibition was described for mammalian PLCδ1, which binds PIP2-containing membranes via its pleckstrin homology domain. The IP3 produced following receptor-stimulated
activation of PLCδ1 antagonizes its binding to PIP₂, causing translocation of PLCδ1 from the plasma membrane to the cytosol and rendering it inactive (35, 36).

Common phenotypic defects of Δarg1 and Δplc1 including sterility, compromised cell wall, thermosensitivity, reduced capsule size and melanization, may be caused by accumulation of PIP₂ or absence of complex IPs. In *S. cerevisiae*, the lack of detectable PP-IPs in Δarg82 and Δkcs1 is thought to be responsible for vacuolar fragmentation, salt stress sensitivity and a defective cell wall (22). However, excessive accumulation of PIP₂ is also detrimental, as deletion of PIP₂ dephosphorylating synaptojanins in *S. cerevisiae* caused defects in actin organization, endocytosis, and clathrin-mediated sorting between the Golgi and endosomes (21). It should be noted that, despite the overall phenotypic similarity between *C. neoformans* Δplc1 and Δarg1, the extent of each defect was not the same. Δarg1 was more sensitive than Δplc1 to a growth temperature of 37°C and did not produce any detectable melanin, while Δplc1 was slightly pigmented. Virulence of Δplc1 and the propagation in moth larvae were markedly reduced, while Δarg1 propagated and caused insect death, albeit more slowly than WT. These differences may be attributed to the opposing trend in IP₃ content in the two mutants and/or the more significant accumulation of PIP₂ in Δarg1.

Consistent with defects in pathogenicity-related phenotypes, virulence of both Δplc1 and Δarg1 was attenuated in *G. mellonella*, which is a proven invertebrate model of cryptococcosis (37). Larvae infected with Δplc1 survived longer than larvae infected with Δarg1, despite Δplc1 having a faster growth rate than Δarg1 at 30°C *in vitro* on a standard growth medium. This difference in survival correlated with the extent of propagation of each strain within the host: while both WT and Δarg1 underwent significant propagation, Δplc1 did not. Differences in the ability of Δplc1 and Δarg1 to assimilate nutrients and/or maintain viability within the host may
be responsible for the difference in the virulence between these mutants, as the nutritional
environment in larval hemocoel differs significantly to that of standard liquid media used to
propagate yeast in vitro. In support of this hypothesis, ScΔplc1 exhibits compromised ability to
assimilate galactose, raffinose and glycerol (5) and rapidly loses viability in the absence of a
nitrogen source (5).

As Plc1-mediated hydrolysis of PIP₂ also generates DAG, it is possible that some of the
phenotypic abnormalities of Δplc1 are caused by the reduction in DAG content. Moreover, if
Plc1 is inhibited in Δarg1 by the excess of IP₃, the same defect might be expected in both
mutants. However, we propose that it is unlikely that a reduction in DAG content in both mutants
is responsible for their phenotypic similarity: firstly, as S. cerevisiae Δplc1, Δarg1 and Δkcs1
share a significant degree of similarity, the absence of PP-IPs is more likely to be the major
cause of common phenotypic defects in these mutants (38, 39). Secondly, PLCs is not the only
source of DAG in C. neoformans: DAG is also produced by Ipc1 (inositol-phosphorylceramide
synthase-1), an enzyme of the sphingolipid biosynthetic pathway. Ipc1-derived DAG was shown
to facilitate C. neoformans protein kinase C activation and subsequent melanization (40, 41).

In S. cerevisiae, the absence of Plc1, Arg82 and Kcs1 caused an increase in cell size and
vacuolar fragmentation. Although the cells of the C. neoformans Δarg1 mutant were also
enlarged, we observed an opposite defect in vacuolar morphology: one large vacuole dominated
the mutant cells, in contrast to multiple small vacuoles observed in WT. This defect was
significantly more pronounced in Δarg1 than in Δplc1. As PIP₂ plays an essential role in vacuole
fusion in S. cerevisiae (42), elevated PIP₂ in C. neoformans Δarg1 and, to a lesser extent, in
Δplc1, may be responsible for the enlarged vacuoles in these mutants.
We observed a dramatic cell separation defect in both Δplc1 and Δarg1. Post-mitotic mother and daughter cells remained fused, often forming new buds. Interestingly, a similar defect was observed in an S. pombe Δipk1 mutant, but not in S. cerevisiae (25). S. pombe Δipk1 cells were defective in dissolution of the septum and the cell wall surrounding the septum, resulting in accumulation of binuclear cells with a medial division septum. This defect was attributed to the absence of IP6 and IP7 in S. pombe Δipk1, a feature likely to be shared by C. neoformans Δplc1 and Δarg1. As phosphoinositides and IPs are involved in multiple aspects of intracellular trafficking, we tracked the endocytic pathway in C. neoformans using the lipophilic styryl dye, FM4-64. Endocytosis is initiated at the plasma membrane which invaginates to form an endocytic vesicle. The endocytic vesicle is then transferred to an endosomal compartment that subsequently fuses with vacuoles. However, in the S. cerevisiae Δarg1 and Δkcs1 mutants, FM4-64 accumulated in aberrant endosomal intermediates juxtaposed to vacuoles (23). In contrast, the C. neoformans Δarg1 mutant accumulated FM4-64 in large vacuoles, which were typical for this mutant, but rarely appeared in wild type cells. Interestingly, the internalization of FM4-64 was considerably slower in Aplc1, as compared to wild type and Δarg1. The striking differences in vacuolar morphology and endocytosis between S. cerevisiae Δarg82 and C. neoformans Δarg1, despite the similarity of their IP3/PIP2 profiles, imply difference in the regulation of vacuole fusion and/or intracellular trafficking in pathogenic basidiomycetes such as C. neoformans and the non-pathogenic ascomycete, S. cerevisiae.

In mammalian cells, Plc1-derived IP3 serves as a precursor for complex IPs, but it also triggers calcium release and subsequent calcineurin activation. Yeast, however, do not possess orthologs of mammalian IP3 receptors, and the ability of PLC to activate calcineurin in yeast is unclear. Limited phenotypic similarity between CnΔplc1 and the calcineurin mutant, Δcna1,
clearly indicates that calcineurin does not depend solely on Plc1 for its activation. We therefore tested the possibility that Plc1 partially contributes to calcineurin activation. Inhibition of calcineurin in Δplc1 at 25°C caused growth retardation, indicating that under conditions that do not normally require calcineurin function, calcineurin is essential to compensate for the absence of Plc1. We previously demonstrated that chitin synthase-encoding gene CHS6 is regulated by calcineurin and highly expressed in the presence of Calcofluor white (12). Although expression of CHS6 was lower in Δplc1 than in wild type, inhibition of calcineurin in Δplc1 caused a dramatic reduction in CHS6 expression, indicating that calcineurin is active in Δplc1. To test whether some of the Δplc1 abnormalities, which are shared by Δcna1, are due to reduced calcineurin activation, we attempted to rescue these defects by providing extracellular calcium, which activates calcineurin in C. neoformans (12). However, none of the Δplc1 defects tested were rectified by extracellular calcium. Taken together, these findings suggest that Plc1 does not contribute significantly to calcineurin activity. Similar to Plc1 and Cna1, Arg1 is essential for growth at 37°C, formation of mating filaments and cell wall integrity. It is therefore likely that the Plc1/IPK pathway functions in parallel to calcineurin to regulate these essential functions, although there may potentially be a cross-talk between the two pathways. This redundancy most likely contributes to the robustness of C. neoformans and its success as a pathogen.

In summary, our study shows that C. neoformans Plc1 produces IP3, which serves as a precursor for the synthesis of more complex IPs by the inositol polyphosphate multikinase, Arg1. Arg1-generated IPs are likely to be further phosphorylated, as the C. neoformans genome encodes inositol hexa- and heptakisphosphate kinases. Plc1, Arg1 and their products play multiple roles in cellular homeostasis and have a dramatic impact on virulence of C. neoformans. The role of IPKs and their products has never been addressed in pathogenic fungi and our
findings lay the foundation for investigating the mechanisms by which IPKs and IP metabolism promote fungal virulence.

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References


FIGURE LEGENDS

Figure 1. Recombinant CnPlc1 preferentially hydrolyses PIP$_2$. (A) Expression of HIS-tagged CnPLC1 in S. cerevisiae. CnPLC1-expressing and control strains were grown under non-inducing (glucose) and inducing (galactose) conditions. Lysates were subjected to SDS PAGE and Western blotting with antibodies against the Histidine tag. Sacch-EV, empty pYES2/NT vector; Sacch-LacZ, β-galactosidase-expressing positive control; Sacch-CnPLC1 (1) and (2), two CnPLC1-expressing recombinants. (B) Purified CnPlc1 and crude lysates were assayed for hydrolytic activity against PI or PIP$_2$, and release of $^3$H-inositol from each substrate was measured by β-counting. Results represent the mean ± SE (n ≥ 3). * represents a statistically-significant difference relative to Sacch-CnPlc1 lysate and Nickel affinity (His)-purified Plc1 (p-value < 0.001, using a two-tailed t-test). # represents a statistically-significant difference relative to Nickel affinity (His)-purified Plc1 (p-value = 0.01).

Figure 2. Quantification of IP$_3$ (A) and PIP$_2$ (B) in Δplc1, Δarg1 and WT. Results represent the mean and standard deviation of three IP$_3$ measurements. The differences in IP$_3$ and PIP$_2$ content between any two strains are statistically significant (P<0.01 using two-tailed t-test). Each experiment was performed 3 times with similar results. In (A) and (B) IP$_3$ was measured using the IP$_3$ assay kit. In (B) membrane-associated PIP$_2$ was quantified indirectly by measuring IP$_3$ released by alkaline hydrolysis.

Figure 3. Phenotypic characterization of Δarg1, as compared to Δplc1, WT and Δarg1:ARG1 (A) WT H99, Δarg1, Δarg1:ARG1 and Δplc1 (serially-dilated 10-fold, 10$^6$-10$^7$).
cells/spot from left to right) were spotted on YPD plates and incubated at 30°C or 37°C, and onto a Congo red-supplemented plate to assess cell wall integrity at 30°C. (B) Melanization of WT H99 and the mutant strains was compared on minimal medium supplemented with the laccase substrate, L-DOPA. (C) WT H99, Δarg1, Δarg1:ARG1 and Δplc1 (MATα) were crossed with KN99 (MATα) on V8 agar plates and incubated for 10 days to assess formation of mating filaments. (D) Wild type and mutant strains were grown in minimal medium (broth) to induce capsule production.

Figure 4. Comparison of the virulence of Δplc1, Δarg1 and WT using a Galleria mellonella infection model. G. mellonella larvae were inoculated with WT and mutant strains as described in the Methods. The health of the larvae was monitored for 10 days and their death recorded.

Figure 5. Vacuolar morphology and cytoskeletal organization in Δplc1, Δarg1 and WT (A) C. neoformans cells grown in YPD were stained with the vacuolar lumen marker, carboxy-DCFDA. Large vacuoles in Δarg1 are indicated by arrowheads. (B) Vacuole size and number per cell were quantified in WT and mutant strains, as indicated. Bars represent the mean and standard error (n=20 cells for each strain). The difference between WT and Δarg1 and between WT and Δplc1 are statistically significant (P<0.05 using a two-tailed Mann-Whitney test). (C) YPD-grown cells were fixed and stained with Alexa Fluor 488-phalloidin (green) to visualize F-actin, and DAPI (blue) to visualize nuclei. Broken arrows indicate multicellular aggregates in Δarg1 and Δplc1. Solid arrows indicate actomyosin rings separating mother and daughter cells.
Figure 6. Tracking endocytosis in Δplc1, Δarg1 and WT with FM4-64 Internalization of the lipophilic dye, FM4-64, was observed immediately after staining (time point 0), and 10, 40 and 60 minutes incubation in fresh medium. Arrows indicate vacuoles labelled with FM4-64.

Figure 7. Addition of exogenous calcium does not rescue the high temperature growth, cell wall and mating defects of Δplc1 (A) Sabouraud agar (SAB) was supplemented with 5 or 50 mM CaCl2. The plates were spotted with WT and Δplc1 cells (serially-diluted 10-fold, 10^6-10^7 cells/spot from left to right) and incubated at 37°C. (B) YPD plates were supplemented with Congo Red and CaCl2 as indicated. Fungal cells were spotted onto the plates as described in (A), and the plates were incubated at 30°C. (C) WT and Δplc1 (MATα) were crossed with KN99 (MATα) on V8 agar plates with or without 5 mM CaCl2, to assess formation of mating filaments.

Figure 8. Inhibition of calcineurin in Δplc1 causes reduced expression of the calcineurin-dependent chitin synthase gene, CHS6, and growth retardation (A) WT, Δplc1 and Δcna1 were grown in YPD broth and incubated for 1 hour with calcofluor white (CFW, 2.2 mg/ml) in the presence or absence of the calcineurin inhibitor, FK506 (10 μg/ml). CHS6 expression was quantified by qRT-PCR and the results are expressed relative to WT + CFW, which has been normalized to 1.00. (B) Cell suspensions of WT H99, Δplc1 and the cell wall integrity mutants Δmpk1 and Δpkc1 were serially diluted 10-fold (10^6-10^7 cells/spot from left to right) and spotted onto YPD plates supplemented with 0.5 M sorbitol (essential for Δpkc1 growth). CFW and FK506 were added as indicated.
Table 1. Primers used in this study.

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