Investigating the role of Ips1 on localization of mRNAs critical for virulence in *Candida albicans*

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Abstract

Candida albicans is an opportunistic fungal pathogen which may cause systemic infection and has been the leading cause of life-threatening invasive infections for decades (Morad et al., 2018). *C. albicans* is polymorphic, where both yeast (typically commensal) and hyphae (associated with tissue invasion and damage) are determinants of pathogenicity in *Candida* (Noble et al., 2017; Mayer et al., 2013). Hyphal growth is dependent on asymmetric distribution of proteins and messenger RNA (mRNA) to the hyphal tip (Elson et al., 2009). *Candida* mRNA transport resembles the *Saccharomyces cerevisiae* She-based transport system, with a She3 ortholog present in *C. albicans*. The protein She2 is essential for nuclear export and mRNA transport in *S. cerevisiae*; however, there is no known *C. albicans* She2 ortholog (Elson et al., 2009). This study tests the hypothesis that Interacting Protein of She3 (Ips1), a protein which copurifies with She3 and whose function is unknown, is necessary for *ASH1* mRNA transport, an mRNA which is known to contribute to hyphae virulence (Inglis et al., 2002). To explore this hypothesis, *C. albicans* strains were used in RNA fluorescence *in situ* hybridization (FISH), and *ASH1* mRNA localization was determined using confocal microscopy and quantified using Fiji ImageJ. Our results suggest Ips1 is critical for *ASH1* mRNA localization, as cells lacking Ips1 showed significantly diminished *ASH1* hyphal tip localization. These data suggest a role for Ips1 in hyphal development and highlight its importance in developing the pathogenicity of *Candida albicans*.

Project Objectives

The primary objective of this study was to optimize the RNA FISH using *ASH1* mRNA probes, to then use this assay to investigate the role of Ips1 in *ASH1* mRNA localization in *C. albicans*. To achieve this goal, I executed the RNA FISH protocol using previously tested knockout strains of *C. albicans*, updating and further developing the working FISH protocol. Furthermore, I developed a protocol for effectively analyzing confocal microscopy results using Fiji ImageJ. Once the FISH protocol was optimized, I introduced our Ips1 knockout mutant *C. albicans* to compare it to wildtype *C. albicans* and other known mutant phenotypes, analyzing results using confocal microscopy images through Fiji ImageJ.

Methodology Used

Hyphal Ca*ASH1* mRNA localization was detected using a set of 41 Stellaris RNA FISH probes (Biosearch Technologies) conjugated to Quasar 570 using a procedure adopted from Lee *et al.* (2016). *Candida albicans* strain AMC 79 and *C. albicans* gene deletion mutants *she3Δ/Δ*, *ash1Δ/Δ*, and *ips1Δ/Δ* were used for these experiments. Strains were streaked on YPD plates (1% yeast extract, 2% bactopeptone, 2% dextrose) containing 80µg/mL uridine and incubated at 30°C for 72 hours. Overnight cultures were prepared with YPD broth which contained an added 80µg/mL of uridine. These liquid cultures were incubated overnight at 30°C on a roller. For hyphal induction the following morning, cells were spun down and resuspended in prewarmed HEPES-buffered Gibco™ RPMI 1640 Medium (RPMI; Thermo Fisher Scientific), before being transferred to prewarmed flasks containing RPMI with 80µg/mL of uridine. Fungi were then incubated for 80 minutes at 37°C with shaking to induce hyphae.

Hyphae were collected via centrifugation and fixed using 4% paraformaldehyde. Cells were incubated during fixation for 1 hour at 37°C with shaking. Fixed cells were washed with Buffer B (17 mM monobasic potassium phosphate, 83 mM dibasic potassium phosphate, 1.2 M sorbitol) resuspended in spheroplasting buffer (Buffer B, 2 mM vanadyl ribonucleoside complex (New England Biolabs), 20 mM β-mercaptoethanol), and cell walls digested with 750 U/mL lyticase (Sigma-Aldrich). Following lyticase addition, tubes were taped to a roller at 30°C and samples were collected on slides every 10 minutes; treatment was terminated when 80-90% of hyphal mother cells were phase-dark (25-90 minutes; Olympus BX51 fluorescence microscope). Spheroplasts were washed with Buffer B, adhered to poly-L-lysine coated coverslips at 4°C for 30 minutes, then permeabilized with ice-cold ethanol overnight at 4°C.

The following day, coverslips were rehydrated with wash buffer (1X saline-sodium citrate (SSC), 10% formamide) then incubated with prehybridization solution (100 mg/mL dextran sulfate, 1 mg/mL *E. coli* tRNA (Sigma-Aldrich), 2 mM vanadyl ribonucleoside complex (New England Biolab), 0.2 mg/mL bovine serum albumin (Invitrogen), 2X SSC, 10% formamide) at 37° for 30 minutes. Coverslips were incubated overnight at 37°C with hybridization solution (0.75µM probe in prehybridization buffer) in a chamber with humidifying buffer (2X SSC, 100 mg/mL formamide).

Cells were washed with wash buffer at 37°C, 2 x SSC 0.1% Triton X-100 at room temperature, and 1 x SSC at room temperature for 15 minutes each. Coverslips were then incubated with 1X phosphate buffered saline (PBS) with 0.5µg/mL DAPI for 2 minutes and washed with 1X PBS. Coverslips were dried in the dark for 1 hour before being mounted to slides with ProLong Gold antifade reagent (Invitrogen) on each slide. Mounting solution polymerized overnight at room temperature in the dark before visualization. Coverslips were sealed with nail polish the following day.

Slides were analyzed on a Leica 6B confocal microscope in LAS X software using a 63x objective with oil. Images and z-stacks were taken at 1024 x 1024 pixels and images and analysis shown were performed blind. Z-stacks were analyzed using Fiji ImageJ v2.14.0/1.54p software. Samples were compiled into max projections, then using the Cy3 channel, maximum values were obtained to find potential signal. Signals in post-mitotic hyphal cells were compiled, and percentages were obtained for *ASH1* mRNA tip localization.

Results Obtained

Verification steps throughout the experiment were optimized and proved to be useful tools. Hemocytometer counts revealed consistent cell counts prior to hyphal induction. Visualization following hyphal induction showed hyphal formation in all strains. Visualization during lyticase treatment proved to be challenging, resulting in blinding the experiment. During confocal microscopy, distinct hyphae were visualized, and DAPI staining allowed for identification of post-mitotic hyphae during analysis. In wildtype post-mitotic hyphae, the brightest *ASH1* mRNA signal colocalized with the hyphal tip (Figure 1, arrowheads) and with DAPI staining of daughter nuclei (Figure 1, arrows). Nuclear transcriptional sites – colocalization of DAPI stain and *ASH1* mRNA signal – were also visualized in post-mitotic hyphae lacking She3 (*she3Δ/Δ*) or Ips1 (*ips1Δ/Δ*) (Figure 1, arrows). Tip enrichment was defined by an *ASH1* maximum within 2µm of the hyphal tip. While 72% of wildtype cells displayed *ASH1* tip enrichment after 80 minutes of hyphal induction, less than 4% of *she3Δ/Δ* hyphae exhibited *ASH1* tip-enrichment. These data align with previous results (Beane & Wang, unpublished results). Our preliminary results show 4.2% of *ips1Δ/Δ* hyphae exhibited *ASH1* tip-enrichment, exhibiting a phenotype remarkably like that of *she3Δ/Δ*.

The negative control, *ash1Δ/Δ*, exhibited 0% tip enrichment, yielding 0 post-mitotic cells with measurable *ASH1* signal. This result confirms the *ASH1* probe set is specific to *ASH1* mRNA.

Significance and Interpretation of Results

Diminished tip enrichment is seen in *ips1Δ/Δ* compared to wildtype. This phenotype suggests Ips1 is involved in mRNA transport and hinders *ASH1* mRNA localization at the hyphal tip. Furthermore, deletion of She3, a protein essential for asymmetric mRNA localization, prevents mRNA transport in *C. albicans* and exhibits extremely low levels of *ASH1* mRNA localization at the hyphal tip. Previous research in the McBride lab identified an RNA-binding protein (Slr1) whose deletion prevented hyphal tip accumulation of *ASH1* mRNA to a lesser extent (20% hyphal tip localization; Beane et al., unpublished) Because *ips1Δ/Δ* demonstrates a phenotype resembling *she3Δ/Δ*, this suggests Ips1 deletion prevents mRNA localization at the hyphal tip. Our preliminary results suggest Ips1 plays a comparably critical role in mRNA localization in *C. albicans*. As previous work suggests, Ips1 accumulates in the hyphal tip later in hyphal development, suggesting Ips1 helps to accumulate *ASH1* mRNA later in hyphal development as well (Wang, 2022; Elson et al., 2009).

To continue this study of the role of Ips1 in mRNA transport, several further assays could be conducted, as well as replication of this experiment. This experiment will be performed using RNA FISH probes for *RBT4* mRNA, another RNA implicated in *C. albicans*' virulence. This experiment will assess the mRNA interaction specificity of Ips1. Further, studies should be conducted to identify RNAs bound to Ips1. Together, these studies will clarify the extent of Ips1's role in mRNA transport and its potential contribution to *C. albicans*' pathogenicity.

Figures/Charts

	Post Mitotic Cells with ASH1 mRNA signal		
	Total Number of Cells	**Cells with ASH1 mRNA at the tip**	**ASH1 mRNA at the hyphal tip %**
WT	75	54	72.0
***she3*Δ/Δ**	59	1	1.7
***ips1*Δ/Δ**	40	2	5.0
***ash1*Δ/Δ**	0	0	0.0

Table 1. Percentage of hyphal tip accumulation varies by strain. Signal was detected by threshold values from images without probe. A hyphal signal was recorded for all post-mitotic cells. Tip enriched percentages were determined by dividing post-mitotic cell counts by all cells counted.

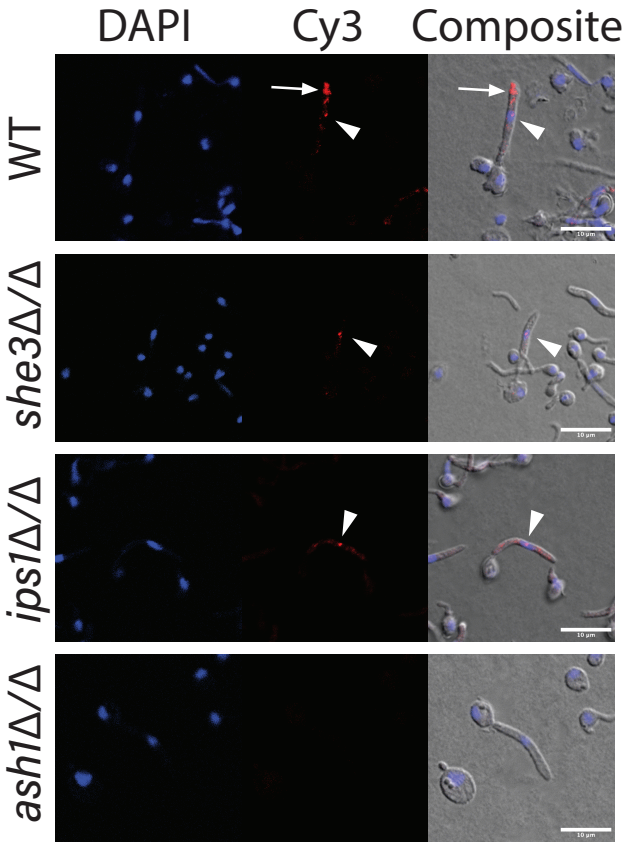

Figure 1. ips1Δ/Δ prevents *ASH1* hyphal tip accumulation. Hyphae were induced at 37°C and cells were fixed with paraformaldehyde. Cell walls were digested and mRNA probe was hybridized to cells. Images were taken using the Leica Confocal Microscope. Arrows indicate enriched tip signal; arrowheads indicate daughter nuclei signal.

Acknowledgements and References

I would like to thank my mentor, Professor Anne McBride, for allowing me to work in her research laboratory, for teaching me necessary laboratory techniques, and for training me on Bowdoin's confocal microscope. I would also like to thank my current lab mates, in particular, Blythe Thompson '26 for her assistance, humor, and cautiousness with the complexities of our project. Finally, I would like to thank the Maine Space Grant Consortium for funding my work this summer. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Aeronautics and Space Administration or of the Maine Space Grant Consortium. Bowdoin College is an affiliate of the Maine Space Grant Consortium.

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