CHARACTERIZATION OF DNA POLYMERASE GAMMA IN THE BASIDIOMYCETOUS YEAST, *CRYPTOCOCCUS NEOFORMANS*

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LIST OF TERMS/ABBREVIATIONS

PolG	DNA polymerase gamma
CnPolG	*Cryptococcus neoformans* DNA polymerase gamma homologue
ScPolG	*Saccharomyces cerevisiae* DNA polymerase gamma homologue
PolGA	Human PolG subunit A protein
PolGB	Human PolG subunit B protein
GST	Glutathione S-transferase
ddNTP	Dideoxynucleotide
gDNA	Genomic DNA
cDNA	Complementary DNA
ssDNA	Single-stranded DNA
mtDNA	Mitochondrial DNA
PBS	Phosphate Buffered Saline
YPD	Yeast Extract Peptone Dextrose media
YPG	Yeast Extract Peptone Galactose media
LB	Lysogeny Broth
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS – Poly Acrylamide Gel Electrophoresis
SAP	Shrimp Alkaline Phosphatase Protein

LIST OF ABBREVIATED GENES

ACT1	Actin gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
MIP1	DNA polymerase Gamma gene
CnMIP1	DNA polymerase Gamma gene from *C. neoformans*
ScMIP1	DNA polymerase Gamma gene from *S. cerevisiae*
CTR4	Copper Transporter 4 gene
GST	Glutathione S-transferase gene
URA5	Orotidine 5’ phosphate decarboxylase gene

ABSTRACT

CHARACTERIZATION OF DNA POLYMERASE GAMMA IN THE BASIDIOMYCETOUS YEAST, *CRYPTOCOCCUS NEOFORMANS*

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Cryptococcus neoformans is a common basidiomycetous yeast and an obligate aerobe that inhabits most environments across the world. *C. neoformans* is also an opportunistic pathogen capable of causing a fungal infection of the central nervous system known as cryptococcal meningoencephalitis. This disease is often observed in patients suffering from AIDS or other immune-compromising afflictions. The antifungal medications currently used to treat *C. neoformans* infections are expensive and often incapable of eradicating infection. Understanding vital systems in this organism are critical to the production of cheaper and more effective medications. Mitochondria are a critically important organelle in aerobic organisms, generating more than 90% of cellular ATP. DNA polymerase gamma (PolG) is a nuclear-encoded DNA polymerase that has been shown in many organisms like humans, *Drosophila*, and *Saccharomyces*, to play a vital role in maintaining and replicating mitochondrial DNA. Homology searches have revealed that *C. neoformans* contains a single putative DNA polymerase gamma gene (*CnMIP1*). The aims of this project were to determine the essentiality of this gene, and to characterize the biochemical function of the encoded protein (CnPolG). In order to determine if *CnMIP1* was essential to the survival of this yeast, the gene was knocked

down *in vivo* using RNA interference. This showed that cryptococci are incapable of survival without CnPolG, thus providing evidence of the importance of *CnMIP1* in *C. neoformans* viability. Although DNA sequence homology showed the presence of a polymerase and an exonuclease domain in *CnMIP1*, the function of the encoded protein had to be experimentally determined. For this, the *CnMIP1* gene was cloned and recombinant protein was expressed in both bacterial (pET28 plasmid) and yeast (pRS424 plasmid) expression systems. The bacterially-expressed protein was heavily degraded, but addition of ethanol to the media has shown promising results. The yeast-produced protein, using a codon-optimized gene construct, showed significantly reduced levels of degradation, but this has been an unreliable expression system for large-scale production of this protein thus far. The polymerization ability of recombinant CnPolG purified from bacteria has been assayed using M13 ssDNA as template. Additionally, it has been shown that this protein can utilize either tailed or untailed primers to initiate polymerization and performs more robustly in a low salt environment. Finally, these assays have also demonstrated that polymerase activity is dependent on Mg^{2+} or Mn^{2+} ions and that functionality increases when both cations are present. Comparing homologues from *Saccharomyces*, humans, and *Cryptococcus* shows the presence of two domains in CnPolG that are not present in the other two homologues. Moving forward, these domains could provide functional insights into the regulation of mitochondrial polymerases and may serve as potential pharmaceutical targets if they play an essential role in *CnMIP1* functionality.

Chapter I. INTRODUCTION

Cryptococcus neoformans

Fungi belong to the domain Eukarya and include an extremely diverse collection of species ranging from unicellular yeasts to massive systems of mycorrhizae. *Cryptococcus neoformans* is an encapsulated basidiomycetous yeast.[1] It has been isolated from ecosystems across the world and is known to inhabit soil, plants, and animal tissues. It can be commonly found in the single-celled form that is capable of self-replication via budding. It is also capable of forming hyphae which allows for sexual reproduction and formation of basidiospores.[1] *C. neoformans* produces a polysaccharide coat that is unique to the yeast that results in a clear halo around the yeast body when stained with India ink and allows for its rapid identification.[2]

Until recently, *C. neoformans* existed as two separate serotypes, A and D and a third hybrid serotype AD. These were determined via analysis of reactivity with various polyclonal antibody serums.[2] Serotypes B and C were also defined in this study, and eventually grouped as a separate species, *Cryptococcus gattii*. *C. neoformans* A and D serotypes have recently undergone changes in classification again. What was formerly considered *C. neoformans* variety *neoformans* serotype D is now considered its own species, *C. deneoformans*.[3] In this thesis I will be using both *C. neoformans* and *C. deneoformans* for different experiments.

C. neoformans is a common opportunistic pathogen in individuals suffering from acquired immunodeficiency syndrome (AIDS) or individuals with otherwise suppressed immune systems.[2] In rare cases, it has been documented to have infected individuals with fully functional immune systems as well. Rates of *C. neoformans* infection increased rapidly with the rise of

AIDS[4] and the adoption of cancer treatments involving severe immunosuppression of the patient.[5] Currently, treatments vary depending on the mode of infection. *C. neoformans* infections are generally treated over a 2 to 4-week period with Amphotericin B deoxycholate and Flucytosine, and extended treatment with Fluconazole. Fluconazole treatments range from 8 weeks to continuous treatment for the duration of the infected individual's lifetime.[4] This is expensive, and components of the treatment have proved potentially toxic, especially in patients with renal conditions.[6] The complicated nature of these treatments and potential for dramatic side effects have driven researchers to probe the genome of *C. neoformans* for essential genes with novel domains that could serve as potential targets for pharmaceutical intervention. Currently, the development of designer peptides which can inhibit proteins with high specificity is making significant headway,[7] so identifying essential proteins has more potential than ever to generate new pharmaceutical targets.

Mitochondria

Mitochondria are essential organelles in obligate aerobes. These organelles are unique in that they contain their own DNA (mtDNA). As a result, the mitochondria are equipped with all the necessary proteins for replication and repair of the mitochondrial genome. Attempts to explain the presence of self-replicating DNA outside of the nucleus have bolstered the theory of endosymbiosis.[8] This theory states that mitochondria most-likely arose from an obligate intracellular parasite which became a mutualistic symbiosis. Over millions of years this relationship grew so intimate that the intracellular species eventually became completely dependent on its host for metabolism. In fact, the majority of the proteins and RNAs localized to the mitochondria are encoded by genes found in the nucleus. Of more than 1000 proteins found in human mitochondria, only 13 are encoded by mitochondrial DNA.[9] This means that over time

many of the genes necessary for maintenance of the mitochondria were translocated from mtDNA to nuclear DNA. Why most genes were transferred to the nucleus while others remain as mtDNA is unclear, but it is thought that some of the mtDNA gene products may simply be too hydrophobic, or too large, to cross the mitochondrial membrane.[10]

The mitochondrial genome is much smaller than the nuclear genome,[11] and mtDNA is packaged as protein-DNA constructs known as 'nucleoids'. These nucleoids are attached to the inner mitochondrial membrane and harbor multiple copies of the mtDNA genome each.[12] mtDNA is different from nuclear DNA in that it is not bound to histones but instead to the inner mitochondrial membrane.[13] Without the protection of histones, and since it is stored in the same mitochondrial compartment where many reactive oxygen species are generated by cellular respiration, mtDNA is subject to up to 10 times as many point mutation events as nuclear DNA.[14] Accumulation of mutations in the mtDNA is an important factor in aging[15] and many mitochondrial diseases are associated with mutations of mtDNA.[11]

mtDNA exists in a circular form in some organisms, or it is contained as concatemers of genome units in others.[12] Two theories exist on how the DNA is replicated. One states that the mtDNA is replicated at multiple 300 bp origin of replication sites in a bidirectional manner. The other states that a rolling circle mechanism is employed which creates long tandemly repeated mtDNA molecules.[16]

DNA Polymerase Gamma

DNA Polymerase Gamma (PolG) is a conserved protein found in all organisms that utilize mitochondria. The protein is nuclear encoded and belongs to the Family A polymerases[17] which include bacterial polymerases such as DNA Polymerase I from *Escherishia coli*.

Interestingly, the most closely related polymerase is T7 bacteriophage polymerase.[18] Human PolG was first associated with mtDNA in the early 1970s[19] and was identified to be localized to the mitochondria within the decade.[20] The protein was determined to contain a 25 amino acid mitochondrial targeting sequence[21] and localization studies eventually determined that PolG exclusively localized to mitochondria.[22]

The PolG protein is essential in the replication and maintenance processes of the entire mitochondrial genome. Evidence supporting this claim has been provided by studies in multiple model organisms. *S. cerevisiae* was determined to carry a homologous PolG-producing gene, *ScMIP1*, in its nuclear DNA in 1989.[23,24] Studies involving the inactivation of *ScMIP1* led to complete loss of mtDNA and the formation of petite *S. cerevisiae* colonies. Furthermore, point mutations of critical residues within the exonuclease domain of ScPolG resulted in massive increase of mtDNA point mutations.[25] When similar experiments were performed in mice, their rate of aging was rapidly accelerated.[26] In human cell lines, PolG mutants lacking polymerase activity lead to significant mtDNA depletion.[22] Depletion of mtDNA has been associated with mitochondrial disease including Alpers-Huttenlocher syndrome, progressive external ophalmoplegia, hepatocerebral syndromes and others.[15,27]

In humans, PolG is a heterotrimeric holoenzyme composed of three subunits, a 140 kDa polypeptide (PolGA) which is responsible for 5' -> 3'polymerase activity and 3' –> 5' proofreading exonuclease activity, and a 54 kDa homodimeric polypeptide (PolGB) which increases polymerization rate and decreases exonuclease activity.[28]

Human PolGA contains four conserved domains including an N-terminal domain that is essential to polymerase function, a linker domain that aids in DNA binding and associates with

the PolGB subunit, a polymerase domain responsible for DNA synthesis, and an exonuclease domain that performs 3' –> 5' exonuclease activity.

PolGB exists as a homodimer, but only one of the monomers associates with the PolGA subunit in the absence of DNA.[29] While it has been well established that this subunit plays a role in increasing the processivity of PolGA,[30] it has also been shown to increase the DNA affinity of the holoenzyme.[18] PolGB provides a protective effect against enzyme inhibition of PolGA by N-ethylmaleimide[31] and reactive oxygen species.[32] The PolGB subunit has also displayed the ability to increase the relatively low salt tolerance of PolGA.[30]

Polypeptide alignment with sequences from human, rat, fruit fly and yeast show three conserved polymerase motifs and three conserved exonuclease motifs.[17] These motifs include two conserved aspartic acid residues (D890 and D1135) in the first and third polymerase motifs, and a conserved aspartic acid and glutamic acid (D198 and E200) in the first exonuclease domain.[24] These four residues are responsible for coordinating divalent metal cations necessary for polymerase and exonuclease activity. Mg^{2+} is the preferred cation when using DNA as a template while Mn^{2+} is the preferred cation when utilizing RNA templates.[18]

Unlike human PolG, there is only a single 143 kDa subunit for PolG in *S. cerevisiae*. Even without the help of a processivity subunit, this enzyme is capable of similarly high processivity that the human holoenzyme is capable of.[33]

Two different mechanisms have been proposed for mtDNA synthesis by PolG. Synchronous DNA synthesis in which leading and lagging ends are replicated simultaneously,[34] and strand displacement in which DNA is synthesized from an origin of replication known as an

O_H until newly synthesized DNA is displaced by the polymerase as polymerization continues to an origin known as a O_L resulting in asymmetric synthesis of the H and L strands.[35]

Human PolG can utilize several oligonucleotide templates including primed DNA, singly primed M13 ssDNA[31] and synthetic RNA.[36] It has also been shown to exhibit reverse RNA transcription[36] and can perform RNA-directed 3' -> 5' DNA excision and RNA-primed DNA synthesis.[36] This wide variety of DNA and RNA related function is indicative of the massive responsibility this protein has in mtDNA repair and maintenance. Interestingly, this protein prefers different cations depending on its template[18] and increases exonuclease activity as salt concentration increases *in vitro*.[18]

Until recently PolG was thought to be the sole DNA polymerase localized to the mitochondria. Now many other polymerases have been associated with mtDNA and mitochondrial localization including Primpol, DNA polymerase Beta, DNA polymerase Zeta, DNA polymerase Eta, and DNA polymerase Theta.[37]

PolG is known to work with multiple accessory proteins in mtDNA replication. These protein include TWINKLE, an ATP dependent mitochondrial helicase,[38] topoisomerase, which unwinds supercoiled DNA, and mitochondrial single-stranded binding protein, which stabilizes single-stranded DNA.[18]

A single *MIP1* homologue has been identified in *C. neoformans* (*CnMIP1*). The Wallen lab has previously purified small amounts of this gene's protein product (CnPolG) from bacteria and used this enzyme to demonstrate polymerase activity *in vitro.* Preliminary RNAi experiments in the Bose lab have suggested that *CnMIP1* could be essential in *C. neoformans*.

The essentiality of CnPolG may suggest it is a good target for pharmaceutical intervention in *C. neoformans* infection.

In this project, I have initiated the characterization of the CnPolG protein. I have confirmed the essentiality of *CnMIP1* using RNAi and have demonstrated that recombinant CnPolG is capable of DNA polymerization using singly primed M13 ssDNA. Using this assay, I have also demonstrated that recombinant CnPolG requires a divalent cation to function, I have shown that high salt concentration reduces polymerase activity, and I have provided evidence that CnPolG does not perform strand displacement DNA synthesis. Additionally, I have developed a better method of expressing recombinant CnPolG in bacteria and I have begun developing a method of expressing and purifying recombinant CnPolG from yeast.

Chapter II. MATERIALS AND METHODS

Growth conditions

E. coli strains grown on LB agar plates were incubated at 37 °C. *E. coli* strains grown in LB broth were incubated at 37 °C with shaking at 220 rpm. Fungal species grown on YPD, -TRP, or 5-FOA agar plates were incubated at 30 °C. Fungal species grown in broth were incubated at 30 °C with shaking at 220 rpm.

Gene sequence acquisition

Gene sequences were obtained from FungiDB.org release 46. *CnMIP1* sequence ID is CNAG_06769.

Genomic DNA preparation

C. neoformans genomic DNA was obtained using IBCN02. A 5 ml aliquot of YPD broth was inoculated and grown overnight at 30°C with shaking. The cells were harvested by centrifugation at 12,000 g. They were resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl, pH 8; 20 mM EDTA; 1% SDS) and bead beaten for 2-minute increments with 1-minute incubations on ice between the bead beatings. This was repeated until 50% - 80% of the cells were able to be identified as broken under a light microscope. The tubes were then incubated at 70 °C for 10 minutes, thoroughly vortexed and treated with 200 µl of potassium acetate and 150 µl of 5 M NaCl. The solution was mixed by inverting and incubated on ice for 20 minutes before being centrifuged for 20 minutes at 14,500 rpm at room temperature. The supernatant was transferred to a fresh tube, diluted with 450 µl of chloroform and centrifuged for 10 minutes at

14,500 rpm at room temperature. The upper aqueous layer was transferred to a new tube and 200 µl of 30% PEG8000 was added before incubating on ice for 10 minutes. This solution was centrifuged for 10 minutes at 14,500 rpm at room temperature. The supernatant was removed, and the pellet was resuspended in 50 µl of sterile water. 1 µl of 500 µg/ml RNase was added, and the solution was incubated at 37 °C for 30 minutes. 5 µl of 3 M sodium acetate and 150 µl of 100% ethanol were added to the solution. The solution was frozen at -20 °C for 30 minutes. Finally, the solution was centrifuged for 10 minutes at 4 °C, the supernatant was removed, and the pellet was resuspended in 50 µl of sterile water. This DNA sample was stored at -20 °C.

RNA extraction

Before beginning the RNA extraction process all pipet tips, materials, and centrifuge tubes were autoclaved twice to ensure the absence of RNases. To obtain *C. neoformans* RNA, two 6 ml YPD cultures were inoculated with IBCN64 and grown overnight at 30 °C. 3 ml of each culture was centrifuged at 3,000 rpm for 5 minutes at 4 °C. The cell pellets were resuspended in 750 µl of TRIzol® in screw cap tubes with 0.4 mm glass beads. The samples were bead beaten 8 times for 1-minute intervals spaced apart by 2-minute incubations on ice. The supernatant was collected, the beads were washed with 750 µl of TRIzol®, centrifuged as before and the supernatant was pooled to bring the final volume of supernatant to 1 ml. The tubes were then incubated at room temperature for 10 minutes followed by the addition of 200 µl of chloroform. The samples were briefly vortexed, incubated at room temperature for 5 minutes, and centrifuged for 15 minutes at 12,000 rpm at 4 °C. The top layer was isolated and centrifuged for 10 more minutes at 12,000 rpm at 4 °C. The supernatant was diluted with an equal volume of isopropanol and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4 °C. The remaining supernatant was discarded, and the pellets

washed with 750 µl of 70% ethanol. The tubes were centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was discarded, and the pellets were dried for 20 minutes. The pellets were resuspended in 40 µl of DEPC-treated water. 20 µl of 7.5 M LiCl was added to the tubes and incubated overnight at -20 °C. The next day the samples were centrifuged at 14,000 rpm for 20 minutes at 4 °C. The supernatant was discarded, and the pellets were washed twice with 750 µl of 70% ethanol. The pellets were air dried for 10 minutes and eluted in DEPC-treated water. The final concentrations were measured with a Nanodrop ND-1000 spectrophotometer and stored at -20 °C.

cDNA First Strand DNA Synthesis

Initial attempts to generate cDNA from IBCN64 RNA used the Thermo Scientific Verso cDNA synthesis Kit. 1.1 µl of the 452.5 ng/µl RNA sample was used as the user manual protocol suggests. The cDNA was used as template DNA in a PCR using actin primers (BLO001 and BLO002). This PCR was analyzed alongside of a PCR using the same primers but utilizing gDNA as the template primer. All PCR results from this kit were negative.

Subsequent attempts to isolate cDNA were undertaken using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. 1.1 µl of the 452.5 ng/µl CAP59 RNA sample was used as the user manual protocol suggests. This cDNA was used as template DNA in a PCR using actin primers (BLO001 and BLO002). This PCR was analyzed alongside of a PCR using the same primers utilizing gDNA as the template primer.

Sanger DNA Sequencing

DNA to be sequenced was added to an eight-strip of PCR tubes. These tubes contain 600 ng of the template DNA and 25 pmol of primer in a total volume of 15 µl. These eight strips were sequenced by GENEWIZ.

Transformation of C. deneoformans via electroporation

A 30 ml culture of the IBCN43 was grown overnight in the appropriate growth media. The culture was then diluted to $2x10^6$ cells/ml. The cells were grown to approximately $6x10^6$ cells/ml and harvested via centrifugation at 3000 g at 4 °C. The cells were then resuspended in 50 ml chilled water and pelleted as before twice in order to wash away all growth media. The cells were then resuspended in 50 ml of cold electroporation buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 270 mM sucrose). 200 µl of 1 M DTT was added to the electroporation buffer and the mixture was incubated for 15 minutes on ice. The cells were then pelleted as before and resuspended in 50 ml electroporation buffer. The cells were once again pelleted as before and the residual electroporation buffer was stirred back into the cells before they were transferred to the pre-chilled electroporation cuvette. 60 µl of cells were transferred to each electroporation cuvette and the appropriate DNA was added to the cells and stirred into the cells. The electroporator was set to the following: 0.5 kV, 25 µF and 1000 or ∞ ohms. Ideal pulse lengths or time constants are between 15-25 ms. Samples were then resuspended in YPD broth for 3 hours in order to recover from electroporation before being plated on the appropriate selective media.

Electroporation of JEC21 with the pIBB103 and pIBB103-CnMIP1

The pIBB103 and pIBB103-*CnMIP1* plasmids were linearized by restriction digestion using I-SceI. The reactions contained 20µl of each plasmid DNA, 8µl of 10xCutsmart™ buffer,

2µl of I-SceI, and 50µl of water. The linearized DNA aliquots were purified using Wizard® Plus SV DNA Cleanup Kit (Promega) and resuspended in 35 µl of water. Three electroporations were performed using 5 µl of linearized pIBB103, 4 µl of pIBB103-*CnMIP1*, and 3 µl of water as control. The time constants of these electroporations were 36.3 ms, 30.8 ms and 30 ms respectively. The electroporated cells were plated on YPD-neo plates and allowed to grow for 3 days.

Cloning CnMIP1 cDNA in pTOPO

After isolating cDNA from IBCN64, a PCR reaction was performed using the cDNA as template DNA and BLO179 and BLO180 as primers to amplify the full-length *CnMIP1* gene. The reaction mixture for this PCR reaction contained 1 µl of cDNA template, 1 µl of each primer, 1 µl dNTP, 5 µl of 5x Q5 reaction buffer, 0.25 µl of Q5 polymerase and 15.75 µl of water. The thermocycler program was set to the following: 94 °C 30 s; 30 x (98 °C 10 s, 57 °C 30 s, 72 °C 3 m); 72 °C 15 m. The resulting PCR product was purified using the Wizard® Plus SV DNA Cleanup Kit (Promega). This *CnMIP1* PCR fragment was used in a Zero Blunt Topo reaction in order to clone the gene into pTOPO. This reaction mixture consisted of 2 µl *CnMIP1* cDNA, 1 µl salt solution, 1 µl TOPO vector, and 2 µl of water. This reaction mixture was incubated at room temperature for 15 minutes and subsequently transformed into subcloning efficiency cells. The transformation was carried out using 2 µl of pTOPO reaction mixture in 20 µl of subcloning efficiency cells and plated on LB plates containing 100 µg/ml kanamycin. Resulting colonies were screened for proper insertion of the full-length *CnMIP1* fragment by PCR with primers BLO179 and BLO180. The reaction mixture for this was 1 µl of harvested DNA, 1 µl of BLO179, 1 µl of BLO180, 5 µl of 2x Taq polymerase PCR master mix and 2 µl of water.

T4 ligation of EcoRI digested CnMIP1 from pTOPO and EcoRI digested pRS424

The pRS424 plasmid was obtained from the Wallen lab. 2 µl of this plasmid was transformed into 20 µl of subcloning efficiency cells. The resulting colonies were grown overnight in a 5 ml culture and the plasmids were recovered via column purification using the Wizard® Plus SV Miniprep DNA Purification Kit (Promega). The resulting DNA aliquot was used in a restriction digestion with EcoRI-HF. The reaction mixture used contained 25 µl of pRS424 plasmid, 8 µl of Cutsmart 10x buffer, 3 µl of EcoRI-HF, and 50 µl of water. This reaction mixture was incubated at 37 °C for 5 hours. 2 µl of shrimp alkaline phosphatase (SAP) was added to the reaction mixture and incubated at 37 °C for 35 minutes. The reaction was terminated by incubating the mixture at 65 °C for 15 minutes. The DNA in this reaction mixture was then purified using the Wizard® SV DNA Cleanup Kit (Promega) and eluted in 35 µl of water.

Another restriction digestion was performed using pTOPO-*CnMIP1* as the DNA to be digested. This reaction mixture included 15 µl pTOPO-*CnMIP1* (1), 8 µl of Cutsmart 10x buffer, 3 µl of SmaI and 49 µl of water. This reaction mixture was incubated at 37 °C for 5 hours. 3 µl of EcoRI-HF was then added to the reaction mixture and it was incubated at 37 °C for an additional 5 hours. The reaction mixture was then subjected to gel electrophoresis on a 0.8% agarose gel at 100 V for 30 minutes. The DNA band at 4.5 kb was excised and purified using the Wizard® SV DNA Cleanup Kit (Promega) and eluted in 40 µl of water.

A ligation reaction was performed using the reagents prepared above. This reaction mixture included 2 µl of SAP treated pRS424 and 7 µl of gel purified *CnMIP1*, 2 µl of 10x T4 DNA Ligase buffer, 1 µl of T4 DNA ligase, and 8 µl of water. The reaction mixture was incubated at room temperature for 1 hour and then incubated at 16 °C overnight. The reaction

was terminated by incubating the mixture at 65 °C for 15 minutes. 5 µl of the ligation mixture was used in a transformation with 40 µl of NEB® 5 alpha cells. Twenty of the resulting colonies were grown in 5 ml LB broth with 100 µg/ml ampicillin. The plasmids were harvested from these cells using the Wizard® Plus SV Miniprep DNA Purification Kit (Promega). The resulting DNA aliquots were screened for proper insertion and proper orientation of *CnMIP1* using DraIII. DraIII was used because if the insert was present in the pRS424 vector in the correct orientation, the resulting band would be approximately 500 bp. If the insert was oriented backwards, the resulting band would be approximately 4.5 kb.

Sequencing of pRS424-CnMIP1 #18

The pRS424-*CnMIP1*#18 construct was sequenced using three primers in three independent sequencing reactions. The Exon1F and Exon4R primers were used to confirm the full-length of protein was inserted into the pRS424 vector. The Exon1R primer was used to assess the junction between *CnMIP1* and the N-terminal GST tag. All sequencing reactions were set up as described above.

pRS424-CnMIP1 #18 unwanted segment removal by PCR

To remove the unwanted 70 bp segment from our expression vector, a reverse complementary primer was made of Exon1F (BLO245). A PCR reaction was performed using pRS424-*CnMIP1* #18 as the template with BLO245 and Exon1F. This reaction mixture included 1 µl of 4.8 ng/µl pRS424-*CnMIP1*#18, 1 µl of 1 µM BLO245, 1 µl of 1 µM Exon1F, 0.35 µl of 2000 U/ml Phusion polymerase, 4 µl of 5x Phusion GC buffer, 1 µl of 10 mM dNTPs, and 13.45 µl of water. This reaction resulted in a product of approximately 11kb. The DNA product was excised, and gel purified using the Wizard® Plus SV DNA Cleanup Kit (Promega).

pRS424-CnMIP1 intramolecular HiFi

The linear purified product resulting from the pRS424-*CnMIP1*#18 correction PCR was utilized in a HiFi reaction. The reaction mixture included 3 µl of 31.9 ng/µl PCR product, 5 µl of the 2x NEBuilder HiFi Master Mix, and 2 µl of water. This reaction was incubated at room temperature for 5 minutes. 5 µl of the reaction mixture was transformed into NEB® 5-alpha cells and plated on LB plates with 0.1 mg/ml ampicillin. Resulting colonies were screened using Sal1 and correct sequences were confirmed by sanger sequencing.

Homologous recombination in S. cerevisiae

In order to perform homologous recombination in yeast, a fresh culture of IBF42 was grown overnight. The transformation was initiated by centrifuging 0.5 ml of broth culture in a sterile microcentrifuge tube. The supernatant was discarded before stirring in 100 µg of salmon sperm DNA, 1 µg of PCR product made with BLO256 and BLO257, and 0.5 µg of EcoRI and XhoI digested pRS424 to the cell pellet. 0.5 ml of PLATE solution (40% PEG 3350, 0.1 M LiAc, 10 mM Tris HCl pH 7.5, 1 mM EDTA) were added and the tube was vortexed for 10 seconds. 57 µl of DMSO were added to the solution and vortexed for 2 seconds. The solution was left at room temperature for 15 minutes. The solution was then incubated in a 42 °C water bath for 15 minutes. The solution was centrifuged at 10,000 rpm for 15 seconds. The cell pellet was resuspended in 200 µl of PBS and plated on selective media.

Bacterial Overexpression of recombinant Protein

The pET28a-*CnMIP1* protein expression plasmid was obtained from the Wallen lab. This expression plasmid encodes six histidines which are added to the N-terminus of the CnPolG protein. The protein expression plasmid was transformed into BL21(DE3) cells, and the one

colony of transformed BL21(DE3) cells were grown overnight in 100 ml starter culture of LB with 50 µg/ml kanamycin. 10 ml of the starter culture was used to inoculate six 1-liter cultures of LB with 50 µg/ml kanamycin to an OD_{600} of 0.6. Protein production was then induced by the addition of IPTG to a final concentration of 0.5 mM. After 3 hours of induction at 37 °C, the cells were harvested by centrifugation for 5 min at 6000 rpm in a Sorvall SLA-3000 rotor at 4 °C. Cell pellets were then resuspended in 50 ml of Ni-Buffer A (50 mM Tris, 0.5 M NaCl, 0.1 mM EDTA, 5% glycerol) and stored at -80 °C.

Purification of the Bacterially Expressed Protein

The frozen cells were thawed in a room temperature water bath. The solution was then sonicated on ice for 1-minute intervals 5 times with 1.5-minute rests in between. The solution was then centrifuged at 16000 rpm for 45 minutes at 4 °C in a Sorvall SS-35 rotor. The supernatant was diluted with Ni-Buffer A (50 mM Tris pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 5% glycerol) to 150 ml loaded onto a nickel chromatography column. The protein was eluted with Ni-Buffer B (0.25 M imidazole, 50 mM Tris pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 5% glycerol) and visualized with SDS-PAGE and Coomassie blue staining. The eluted product was equilibrated in 2 liters of 90% Q-Buffer A (20 mM Tris pH 8.0, 1 mM EDTA, 1 mM BME, 10% glycerol) and 10% Q-Buffer B (1 M NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 1 mM BME, 10% glycerol) overnight at room temperature. The equilibrated solution was loaded onto the ion-exchange column and then eluted in 3 ml aliquots starting at 10% Q-Buffer B and ending at 80% Q-Buffer B over 200 ml. The product was examined using SDS-PAGE and Coomassie blue staining. The first three 3 ml fractions of the ion exchange elution were concentrated by centrifugation at 4000 g using an Amicon® Ultra 15 mL Filter.

SDS−PAGE

8% polyacrylamide gels were made as follows: 3.68 ml diH_2O, 2.133 ml 30% acrylamide, 2.027 ml of R buffer (250 mM Tris HCl, 192 mM Glycine, 1% SDS pH 8.3), 80 µl 10% SDS, 80 µl 10% APS, 4.8 µl TEMED were mixed together in a small beaker. This solution was poured into a BioRad gel stacking box and allowed to solidify for 15 minutes. 2.1 ml diH_2O, 0.5 ml 30% acrylamide, 0.38 ml of S buffer (500 mM Tris HCl, 192 mM glycine, 1% SDS), 30 µl 10% SDS, 30 µl 10% APS, 3 µl TEMED were added to another small beaker. This solution was poured on top of the existing solidified gel and a 10 or 15 prong comb was used to produce the loading wells. The wells were loaded with the appropriate solution along with dye to visualize the progress of the electrophoresis. BioRad gel boxes were used at variable voltage settings for variable lengths of time.

Western Blot Analysis.

Proteins or lysates of interest were first separated by SDS−PAGE and then transferred to a PVDF membrane. All antibodies were diluted with TBS-T. The membrane was incubated with a 1:1000 dilution of primary GST antibody (ThermoScientific, Cat # MA4-004) or 1:10,000 dilution of primary His_6 antibody (ThermoScientific, Cat # MA1-21315) overnight at 4 °C with shaking. These incubations were followed with three 5-minute washes with TBS-T and then a 30-minute incubation at room temperature with 1:10,000 dilution of secondary antibody conjugated to horseradish peroxidase. The membrane was once again washed three times for 5 minutes each with TBS-T. The colorimetric substrate was mixed, immediately applied to the membrane, allowed to incubate for 4 minutes and then imaged using a Bio-Rad imager.

A similar method of western blot analysis was also performed using HisProbe™-HRP. This probe replaces the primary antibody incubation step. The HisProbe™-HRP is diluted 1:5000 with TBS-T. It is then poured onto the PVDF membrane and incubated at room temperature for 1 hour. The membrane is washed three times for 5 minutes before incubation with the secondary antibody and colorimetric substrate as above.

Preparation of M13 bacteriophage

M13 ssDNA was isolated from XL-1 blue *E. coli* cells infected with M13 bacteriophage. 30 µl of fresh XL-1 blue cells were streaked on LB agar plates with 10 µg/ml tetracycline and grown overnight. One colony was used to inoculate a 25 ml culture of LB broth with 10 µg/ml tetracycline and grown overnight. 1.5 ml of the starter culture was used to inoculate 150 ml of LB broth with 10 µg/ml tetracycline. After two hours, 15 µl of 1.6 x 10^{10} pfu/ml stock M13 virus was added to the 150 ml culture. The viral infected *E. coli* cells were incubated for 5 hours. The cells were centrifuged at 3000 rpm and the supernatant was removed and filtered with a 0.22 µm filter. The final solution was stored at 4 °C.

Purification of M13 ssDNA

10 g of solid PEG 8000 along with 7.5 g of NaCl were added to filtered M13 supernatant. The solution was stirred gently on ice for 30 minutes. The solution was transferred to 50 ml conical tubes. The tubes were centrifuged at 4500 rpm for 30 minutes. The supernatant was discarded, and each pellet was resuspended into a single 3 ml aliquot of TE. Three volumes of 0.1 M NaOH, 1% SDS solution were added and swirled. 1.5 volumes of 3M potassium acetate pH 5.0 were added and incubated on ice for 30 minutes. The solution was transferred to 1.5 ml Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes in a cold centrifuge. The

supernatant was transferred into fresh conical tubes. 1 volume of cold 95% ethanol was added and allowed to incubate on ice for 30 minutes. The solution was transferred to 1.5 ml Eppendorf tubes and centrifuged at 13000 rpm in cold centrifuge for 20 minutes. The supernatant was poured off and 500 µl of cold 75% ethanol was added. Tubes were inverted once and centrifuged at 13000 rpm for 10 minutes. The supernatant was poured off and the tubes were inverted until all ethanol was evaporated. Once completely dry, 25 µl of TE pH 8.0 was added to each tube and placed at 4 °C overnight to resuspend. DNA quantification was performed with a Nanodrop ND-1000 spectrophotometer.

Annealing Primers to M13 DNA

The annealing reactions were typically set up in 50 µl aliquots containing 1x annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA), 100 nM M13 ssDNA, and 120 nM M13 primer. 1 liter of water was then brought to a boil in a 1-liter beaker. The reaction mixtures were placed in the beaker with a floater and the beaker was allowed to cool to 30 °C.

M13 ssDNA Polymerase assay

40 µl reaction mixtures were made containing 1x assay buffer (50 mM Tris pH 7.5, 25-100 mM NaCl, 1 mM DTT, 100 µg/ml BSA, 1 mM dNTPs, 0-10 mM $MgCl_2$, 0-10 mM $MnCl_2$, 100 nM annealed M13 ssDNA). Each reaction mixture was preheated to 37 °C using a water bath before the enzyme was added. The addition of enzyme began the reaction and the reactions were maintained at 37 °C. At varying time points, 10 µl of the reaction mixture was removed and added 10 µl of 2x quench buffer (50 mM EDTA, 0.2% SDS, 10% glycerol) to stop the reaction. Each quenched sample was then loaded onto a 1% agarose gel with ethidium bromide and ran at 20 V for 16 hours. The gel was imaged using Biorad ChemiDoc MP imager.

Chapter III. RESULTS

I. *Determining the essentiality of CnMIP1 using RNA interference in C. deneoformans*

C. deneoformans is an obligate aerobe that is incapable of survival without its mitochondria. Mitochondria rely on genes encoded by mtDNA to undergo cellular respiration. In other organisms, the protein primarily responsible for replicating and maintaining mtDNA is DNA polymerase gamma. This protein is ultimately derived from the nuclear encoded *CnMIP1* gene, and it is possible to knockdown protein levels by taking advantage of RNA interference, a process that destroys mRNA containing a specific sequence.

A former graduate student in the lab, Joshua Boggs, cloned a segment of the *CnMIP1* coding sequence into the RNAi vector, pIBB103, which is designed to produce double-stranded RNA (dsRNA) using opposing galactose-inducible *GAL7* promoters. The configuration of these promoters causes any DNA sequence between them to be transcribed, producing complementary RNA fragments that can bind to each other to form dsRNA, activating the process of RNAi. The gene of interest was inserted between the two promoters and adjacent to the *URA5* gene fragment (Figure 1).

Figure 1. pIBB103-*MIP1*i vector used for *MIP1* RNAi. The opposing GAL7 promoters (in green) flank a *URA5* fragment (in blue), and unique NdeI and SpeI sites for cloning the gene of interest.

The silencing of *URA5* allows the transformed cells to grow in the presence of 5-FOA. The YPG-neo plates are used as a control to show that the induction of the *GAL7* promoters is not lethal to the cell population in the absence of 5-FOA (see Discussion for possible explanation).

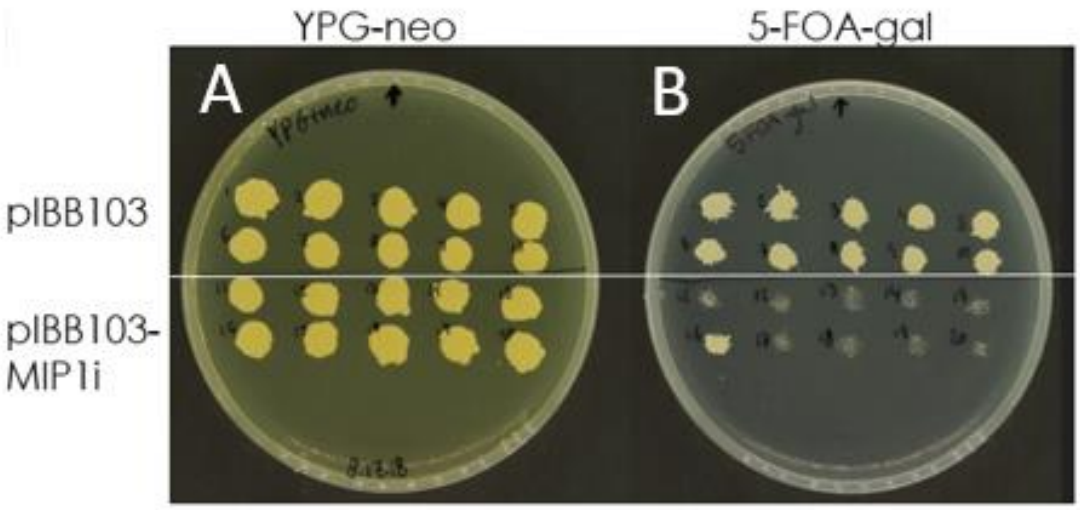


Figure 2. *CnMIP1* RNAi. Growth of pIBB103 (top) and pIBB103-*MIP1i* (bottom) on YPG-neo (A) and 5-FOA-gal-neo (B) media 48h after replica plating.

As seen in Figure 2A, the pIBB103 plasmid allows for growth in the presence of the drug, G418. Once replica plated on 5-FOA-gal the pIBB103 colonies survive while 90% of the pIBB103-*CnMIP1* colonies do not (Figure 2B). This result strongly suggests that *CnMIP1* plays an essential role in *C. neoformans.*

II. *Bacterial expression and purification of CnPolG*

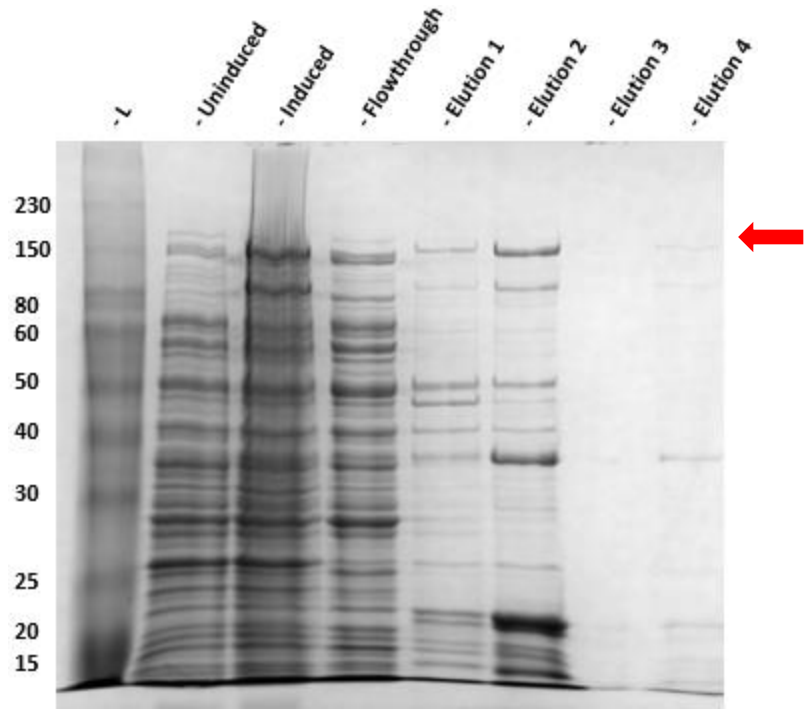
In order to determine the structure and function of CnPolG, large quantities of protein are required. To produce this protein, the Wallen lab generated two plasmids that facilitate the overexpression of CnPolG in *E. coli.* The first plasmid (pET28a-*CnMIP1*) utilizes a 6-histidine (His_6) tag on the N-terminal end of CnPolG to allow purification of the protein by nickel column chromatography. Unfortunately, the majority of the recombinant CnPolG protein expressed using this plasmid was degraded in *E. coli,* resulting in contamination of the final purified protein product with possibly non-functional His_6-tagged CnPolG protein fragments. In order to acquire pure, undegraded CnPolG, a second plasmid (pET28a-*CnMIP1*-His-Strep) was constructed

which tags the recombinant CnPolG with a His_6 tag at the N-terminus and a Strep-tag® at the C-terminus. This allows for full-length protein products to be isolated from degraded products by ensuring the protein contains both the His_6 tag on one end and the Strep-tag® on the other. This plasmid made it possible to isolate full-length CnPolG, but it did so very inefficiently. Using the double-tagged plasmid, low milligram quantities CnPolG were purified from 27 liters of transformed *E. coli* culture. This protein was used in an M13 rolling circle assay to assess its polymerase activity (Figure 13).

II. A *Bacterial expression of CnPolG: in pET28a-CnMIP1induced at 37˚C*

Because very low quantities of protein were able to be isolated using pET28a-*CnMIP1*-His-Strep, optimizing the *E. coli* growth conditions to minimize protein degradation when expressed using the pET28a-*CnMIP1* plasmid was necessary. To set a baseline of expression and purification, the induction protocol that had been used previously by the Wallen lab was followed. Briefly, CnPolG was expressed in *E. coli* using 6 liters of media. Expression was induced with IPTG at 37 ˚C (see Materials and Methods).

A.



B.

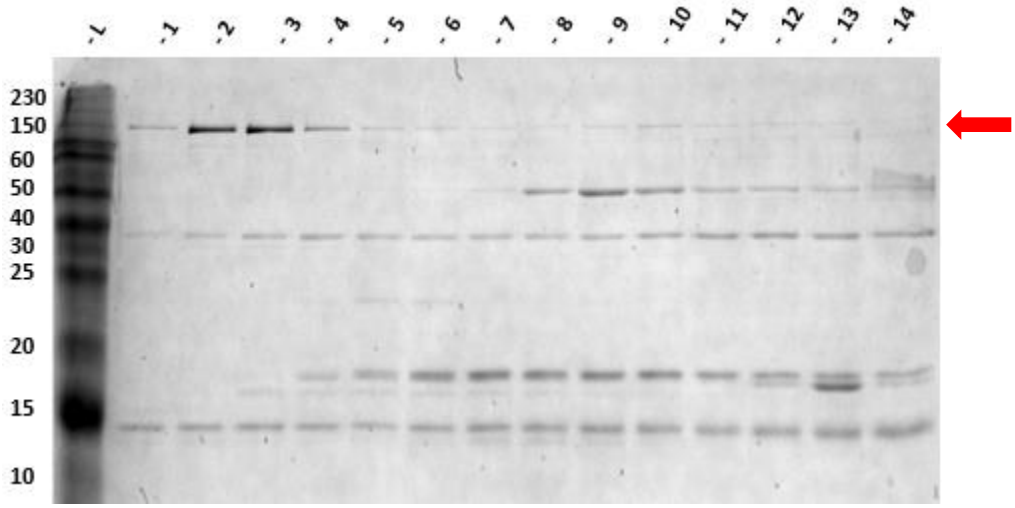


Figure 3. Purification of His_6-tagged CnPolG at 37 °C. (A) Protein profile obtained using a Ni-column. The profile of proteins in uninduced cells, induced cells, flow-through after Ni-column binding, and the progressive elutions with 3 ml of 20 mM imidazole were visualized using an 8% SDS-PAGE gel stained with Coomassie blue. Each lane contains 10 µl of the indicated aliquot. (B) Protein profile obtained using an Ion-Exchange column with increasing salt washes. 10µl of 3 ml aliquots of Ion exchange column elution fractions using increasing NaCl concentration were visualized using an 8% SDS-PAGE gel stained with Coomassie blue. The full-length recombinant protein tagged with His_6 has a mass of 158.7 kDa.

As shown in Figure 3A, an SDS-PAGE gel stained with Coomassie Blue showed the presence of an approximately 158 kDa protein in the induced lane that was not present in the uninduced lane. Since the His_6 tagged CnPolG protein is 158.7 kDa in size, this suggests that the addition of IPTG allowed for production of CnPolG in *E. coli* transformed with the pET28a-*CnMIP1* plasmid. The flow-through lane indicated that very little of the protein product passed over the nickel column without binding. In contrast, the presence of a band of the correct size in the elution 1-4 lanes indicated that full-length CnPolG protein appeared to come off the Ni-column in the presence of 20 mM imidazole. The presence of bands of lower size was most likely due to heavy protein degradation which occurs during expression.

To try and eliminate some of the degraded protein products obtained in the elution from the Ni-column, a second purification step was performed. The GE Life Sciences Hitrap Q 5ml column used for this eluted protein in 3 ml fractions in the presence of increasing NaCl concentration from 100 mM to 800 mM to separate and purify proteins based on charge. A Coomassie blue-stained gel of the fractions (Figure 3B) showed that the majority of the full-length His_6-tagged CnPolG eluted within the first three fractions (or 9 ml). These fractions (1-3) also showed less degraded protein products than that obtained from the Ni-column. These fractions were pooled together, dialyzed overnight, (see Materials and Methods), and then concentrated using an Amicon® Ultra 15 ml filter. The protein that was obtained had a final concentration of 1.65 mg/ml and was later used in functional assays.

II. B *Bacterial expression of CnPolG: in pET28a-CnMIP1 induced in the presence of ethanol*

As expression and purification of CnPolG from the pET28a-*CnMIP1* plasmid at 37 °C did not produce large quantities of undegraded protein, it was decided to explore different conditions which could potentially lessen the rate of protein degradation. One of these conditions

was to grow *E. coli* in the presence of increasing ethanol concentrations before induction of the protein with IPTG.[39] Ethanol is an amphipathic molecule. When exposed to bacterial cell membranes this causes changes in membrane fluidity, membrane transport and membrane lipid composition.[40] This causes increase in DNA synthesis[41] which could contribute to increased protein expression levels in the presence of ethanol.

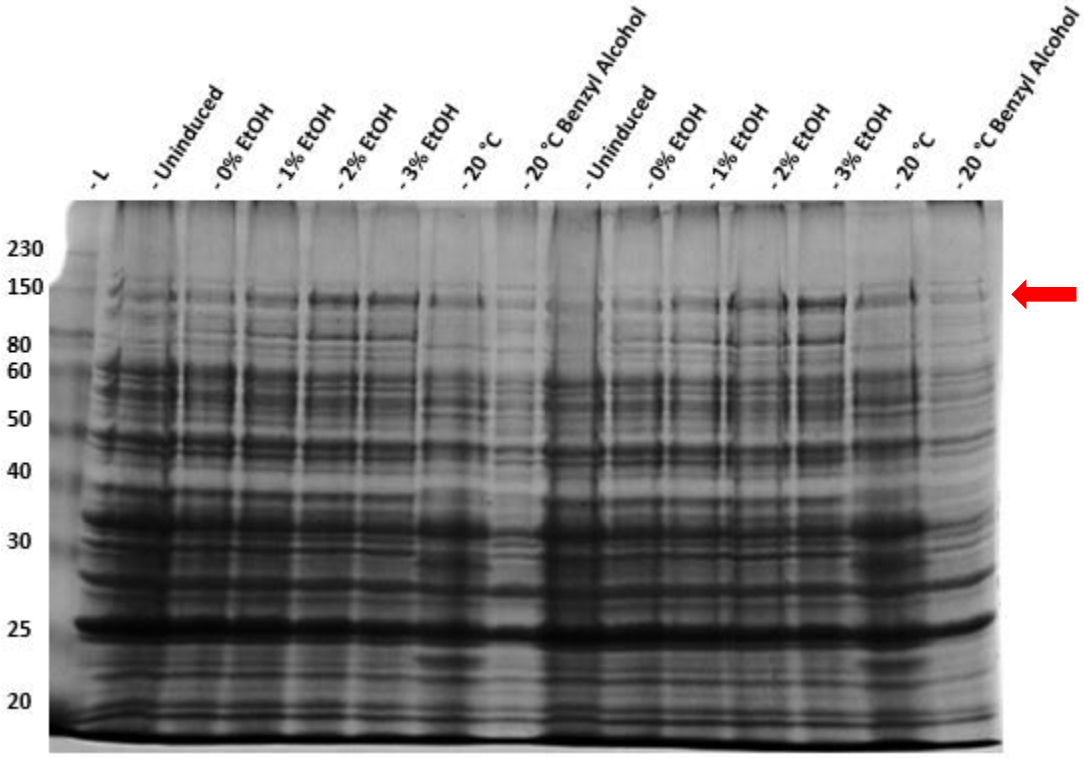


Figure 4. Expression of His_6-tagged CnPolG in the presence of ethanol at 37 °C and benzyl alcohol at 20 °C. Two *E. coli* colonies induced in the presence of ethanol at 37 °C and in the presence of benzyl alcohol at 20 °C visualized by 8% SDS-PAGE stained with Coomassie blue. A band present at approximately 158 kDa indicates protein expression is induced successfully with IPTG. Darkness of this band increases as the concentration of ethanol increases.

Using a protocol developed by Chhetri et. al.,[39] it has been shown that the addition of ethanol to LB+Kan media effectively increased the quantity of protein produced by *E. coli*. Figure 4 showed that the addition of 1% ethanol slightly increased the darkness of a band present at 158 kDa, while the darkness of the bands in the 2% and 3% lanes indicated a dramatic

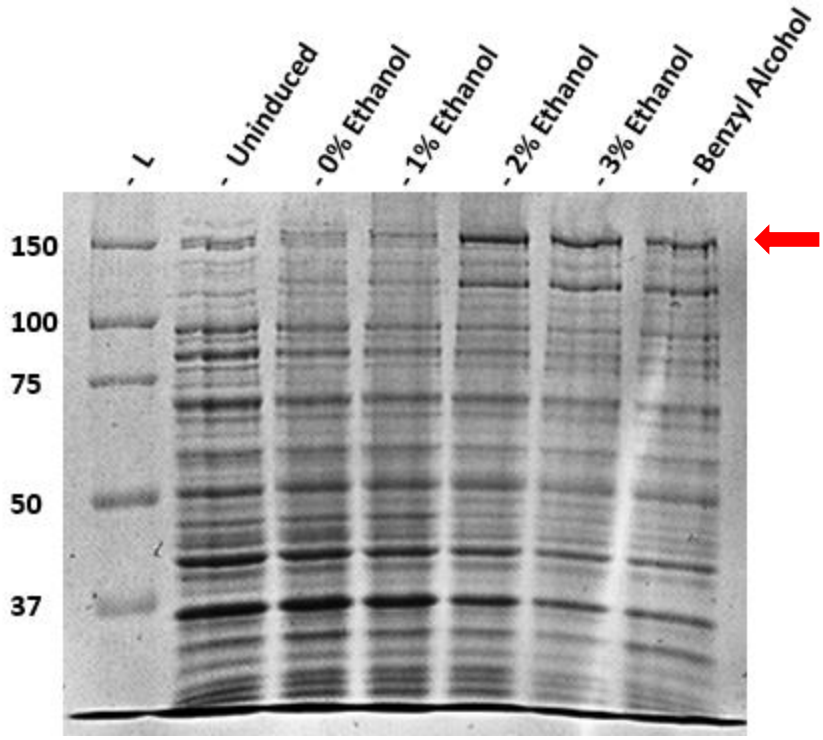
increase the amount of protein produced. This experiment was performed in duplicate in order to ensure the results are reproducible. Western blots utilizing anti-His_6 antibodies were performed using these samples, but failed to produce any result.

To demonstrate the validity of adding ethanol to the *E. coli* growth media, six liters of media with 3% ethanol inoculated with bacteria containing our pET28a-*CnMIP1* plasmid was made. Once the bacteria had been induced, harvested and lysed, the lysate was passed through a nickel column. The resulting elution from this nickel column was void of the CnPolG protein, which may have been due to the age of the nickel column used. Since this result was inconclusive, purification of the protein induced under these growth conditions should be repeated.

II. C *Bacterial expression of CnPolG: in pET28a-CnMIP1-His-Strep induced in the presence of ethanol*

The plasmid pET28a-*CnMIP1*-His-Strep had been used previously to purify small amounts of double-tagged full-length CnPolG protein. Having demonstrated that the addition of ethanol effectively increases the expression level of recombinant protein using pET28a-*CnMIP1*, the same protocol was used with pET28a-*CnMIP1*-His-Strep to determine whether this protocol would increase overall protein expression as well as decrease levels of protein degradation.

A.



B.

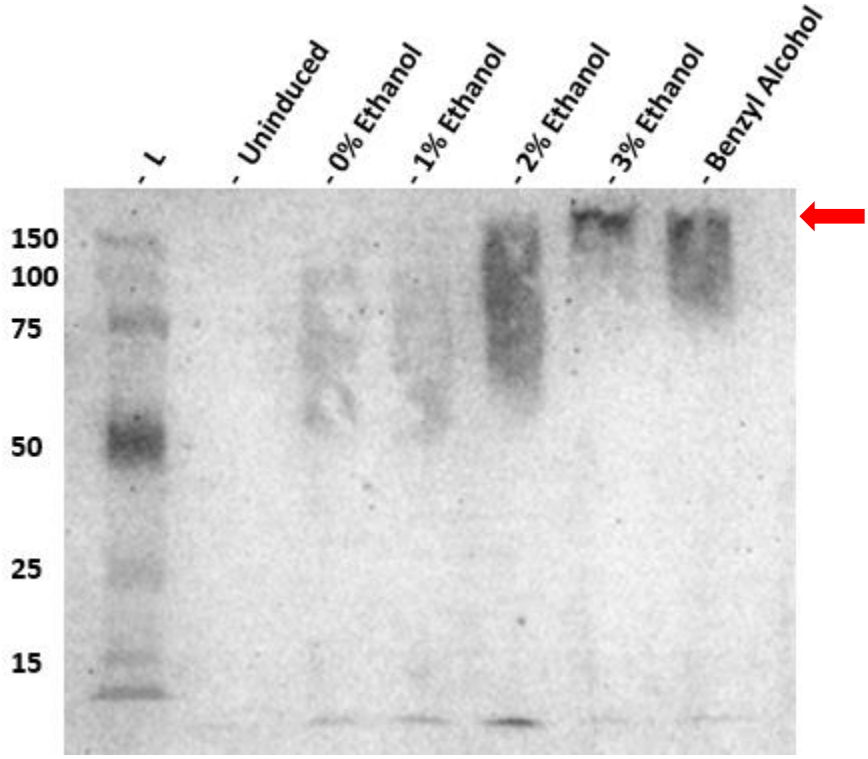


Figure 5. Expression of His_6-tagged/Strep-tagged CnPolG in the presence of ethanol and benzyl alcohol at 37°C. (A) Protein lysate of one *E. coli* colony transformed with *pRS424-CnMIP1*-His-Strep and induced in the presence of ethanol or benzyl alcohol at 37 °C visualized using 8%

SDS-PAGE stained with Coomassie blue. A band present at approximately 158 kDa in the induced lane indicates protein expression is induced successfully with IPTG. Darkness of this band increases as the concentration of ethanol increases. (B) Western blot performed with HisProbe™-HRP conjugate shows that increasing ethanol concentration to 3% has condensed the protein smear into a tight band.

The results of the SDS-PAGE gel (Figure 5A) indicated similar results to the pET28a-*CnMIP1*. A protein band near 158 kDa appeared in the induced lane and increased in darkness at the higher ethanol concentrations, but in the presence of benzyl alcohol seems have diminished this effect. This provided evidence that ethanol could be used to make protein expression more efficient using the pET28a-*CnMIP1*-His-Strep.

The subsequent anti-His_6 western blot (Figure 5B) shows that little protein is induced in the absence of ethanol. There was more His_6-tagged protein of higher molecular mass in the induced samples. There was good expression of the full-length protein and very little degradation (indicated by the absence of smaller His_6-tagged bands) in the protein sample induced in the presence of 3% ethanol. The 3% ethanol lane was also better than the sample induced in the presence of benzyl alcohol. Therefore, it seems that the addition of 3% ethanol to the growth media before induction with IPTG gave the most desirable results when expressing this recombinant protein.

III. *Expression of CnPolG in the yeast, S. cerevisiae*

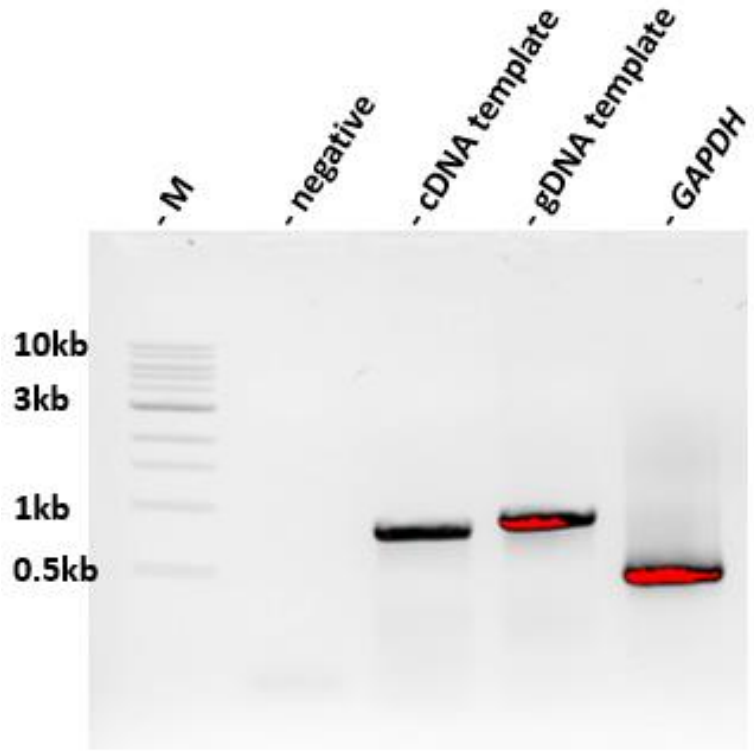
Degradation is common when expressing large, multi-domain proteins in *E. coli*, a prokaryotic organism. In this case, the protein being expressed was a relatively large protein that is native to a eukaryotic organism. Utilizing a more closely related eukaryotic organism for protein expression may reduce or eliminate protein degradation due to the availability of

eukaryotic chaperone proteins. *S. cerevisiae*, a single-celled eukaryote, is commonly used for recombinant protein expression. Here this yeast was used for expression of recombinant CnPolG.

III. A *Obtaining C. neoformans cDNA*

The budding yeast, *S. cerevisiae,* is one of a few eukaryotes that does not efficiently perform intron splicing. Therefore, in order to express CnPolG in this organism, the introns in the *CnMIP1* gene had to be removed by cloning the cDNA version in the yeast plasmid, pRS424. Many problems were encountered during the process of creating the pRS424-*CnMIP1* plasmid. In order to try and circumvent some of these issues, a PCR product of the coding sequence of *CnMIP1* had to be obtained. This was done by using mRNA found in the *C. neoformans* cytosol as a template for reverse transcription. RNA was isolated from *cap59* cells. The extracted RNA was used to generate cDNA using reverse transcriptase (see Materials and Methods). This DNA was used in a PCR reaction to confirm the absence of introns in the cDNA using BLO1 and BLO2 as primers to amplify the *ACT1* gene.

A.



B.

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180   GATTATATATTGGCTATGGAAATGGCTAAATTGGAGGATGGCTATGGCCAGCCTCGAGGT  239
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   GATTATATATTGGCTATGGAAATGGCTAAATTGGAGGATGGCTATGGCCAGCCTCGAG--  238

240   ATGTCTAGGAGATCCCAAAGAAGCTCCAGTAATACTCATATGTTGTCTTCACAGTTAGGA  299
                                                            ||||||
239   ----------------------------------------------------------TTAGGA  244

300   AGATTCGCAAATCCAAATTGCCCTCCCTACATGATCCGCAGTCTTTTCTATGCGATTCGA  359
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
245   AGATTCGCAAATCCAAATTGCCCTCCCTACATGATCCGCAGTCTTTTCTATGCGATTCGA  304

360   CGCAAGCCTCGTCATCCAAGGTTACCTCATCCGCATCACCCACATCACAACCAAGCAGAA  419
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
305   CGCAAGCCTCGTCATCCAAGGTTACCTCATCCGCATCACCCACATCACAACCAAGCAGAA  364

420   AGGGTAAAGAGAAAGAAGTAGTTTCAAATGATTATGCAACGAATGTGCCAAATCAAGATG  479
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
365   AGGGTAAAGAGAAAGAAGTAGTTTCAAATGATTATGCAACGAATGTGCCAAATCAAGATG  424

480   TCCAAACTCTGGAGGATGCCAAACCCATCGACTCGGGGCAAAGCAAGAGCGGTACGTCTT  539
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
425   TCCAAACTCTGGAGGATGCCAAACCCATCGACTCGGGGCAAAGCAAGAGCGG--------  476

540   CGTACAGAACGTCCTCTTTCCATTGCTGATACGAAACCAGGCCCGCGGCGGAATCCTGTT  599
                                                  ||||||||||||||||||
477   ------------------------------------------CCCGCGGCGGAATCCTGTT  495
```

Figure 6. Confirmation of intron absence in *C. neoformans* cDNA. (A) Results of PCR amplification of *ACT1* from cDNA, and gDNA. *ACT1* was amplified using BLO1 and BLO2 to assess the presence of introns from cDNA and gDNA and visualized on a 0.8% agarose gel. Amplification of *GAPDH* served as the positive control using primers from the Revertaid First

Strand DNA Synthesis Kit (ThermoScientific™). (B) Alignment of *C. neoformans* gDNA (top sequence) and Sanger sequencing data of the *C. neoformans* cDNA template (bottom sequence).

The result shown in Figure 6A clearly showed a smaller band in the cDNA lane. The absence of introns was further confirmed via Sanger sequencing, as seen by missing intronic sequence in an alignment of the gDNA (top strand) vs. cDNA (bottom strand) in Figure 6B.

III. B *Making of pTOPO-CnMIP1 from CnMIP1 cDNA*

In order to maintain the cDNA sequence of *CnMIP1*, a Zero Blunt™ TOPO™ cloning kit was used to insert the *CnMIP1* DNA fragment into pTOPO (see Materials and Methods). Approximately 50 colonies were generated using this protocol and six of the colonies were screened for presence of *CnMIP1*. All six of the colonies were confirmed to contain *CnMIP1*. Maintaining this gene sequence allowed for easy amplification and isolation of *CnMIP1* in the future by restriction digestion with EcoRI.

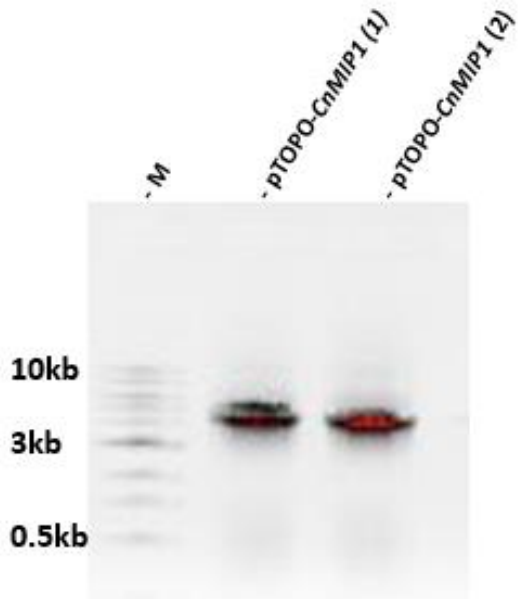

Figure 7. Confirmation of *CnMIP1* in pTOPO-*CnMIP1* by PCR. 0.8% agarose gel containing PCR of pTOPO-*CnMIP1* transformant DNA using BLO179 and BLO180. Full-length *CnMIP1* cDNA is 4359 bp.

Figure 7 represents a PCR used to confirm the presence of *CnMIP1* in two pTOPO plasmids recovered from separate transformant colonies. The presence of a band at 4359 bp indicates that *CnMIP1* has been successfully integrated thus creating pTOPO-*CnMIP1*. Moving forward *CnMIP1* was obtained from this plasmid using EcoRI and XhoI restriction enzymes for use in ligation reactions with pRS424.

III. C *Cloning CnMIP1 cDNA EcoRI site alone of pRS424 vector*

CnMIP1 was isolated from pTOPO-*CnMIP1* by restriction digestion with EcoRI and subsequent gel purification. This DNA was then used in a ligation reaction with pRS424 linearized with EcoRI (see Materials and Methods). The transformant colonies generated by this process were screened using DraIII restriction enzyme. This restriction enzyme was chosen because it could be used to determine the orientation of *CnMIP1* with relation to pRS424. Plasmids which contain the *CnMIP1* gene in the correct orientation would present two DNA bands at 10.8 kb and 500 bp, as seen lane 18 (Figure 8). Plasmids with *CnMIP1* in the incorrect orientation would present two DNA bands of 7.3 kb and 4 kb, as seen in lanes 11 and 20 (Figure 8).

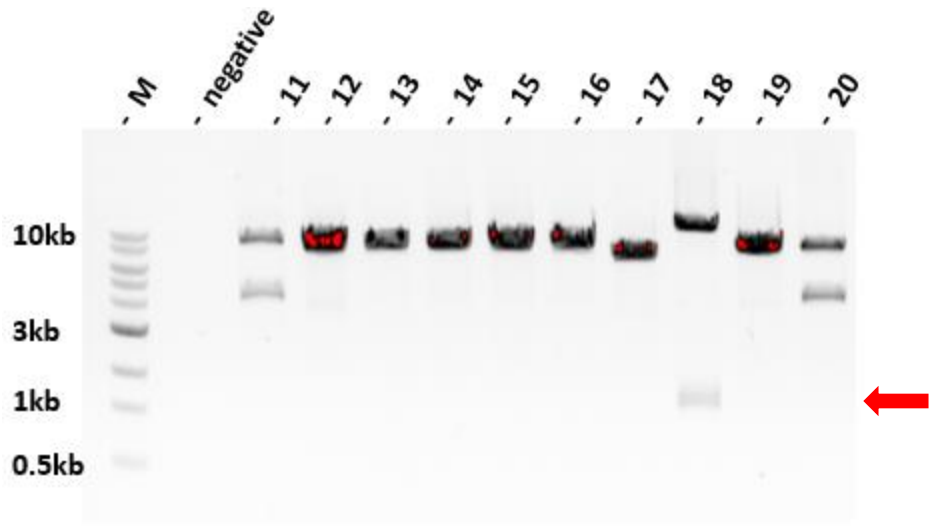

Figure 8. Transformant colony screen for presence of pRS424-*CnMIP1*. Restriction digestion of plasmids harvested from colonies transformed with ligation reaction of EcoRI digested *CnMIP1* and EcoRI digested pRS424 were visualized using 0.8% TAE-agarose gel showing restriction.

Figure 8 reveals that only a single colony (lane 18) out of the 20 that were tested contained *CnMIP1* in pRS424 in the correct orientation. Lanes 11 and 20 contained the insert in the incorrect orientation.

III. D *Yeast expression of GST ScMIP1 and CnMIP1#18*

The pRS424-*CnMIP1*#18 plasmid that was produced allowed for plasmid maintainence and amplification in bacteria, while also allowing recombinant protein to be produced in *S. cerevisiae.* This plamid would also tag the protein with GST to allow column purification and western blotting. The next step was to assess the expression of GST, ScPolG and CnPolG using pRS424, pRS424-*ScMIP1* and pRS424-*CnMIP1*#18 respectively. pRS424-*ScMIP1* was acquired from the Wallen lab.

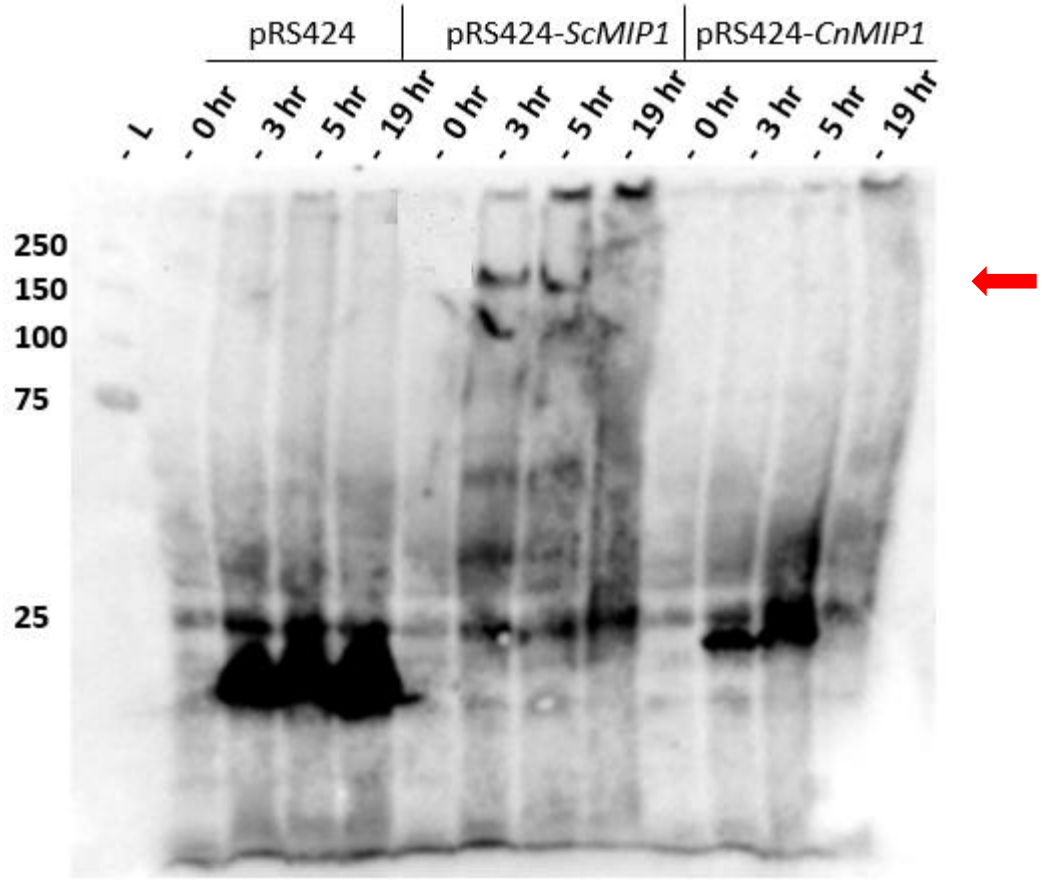

Figure 9. Expression of GST, ScPolG, and CnPolG in *S. cerevisiae* at 30 °C. Western blot was performed with GST antibody. Protein expressed using pRS424, pRS424-*ScMIP1*, and pRS424-*CnMIP1*#18 are represented. A band at 25 kDa is expected from GST, a band at 160 kDa is expected from ScPolG, and a 183 kDa band is expected from CnPolG.

The first attempt to express CnPolG in *S. cerevisiae* produced unexpected results. GST expressed robustly at the expected size at all time points past 0 hours. Small amounts of ScPolG can be observed at the expected size after 3 and 5 hours, but after 19 hours the protein has disappeared. The CnPolG lanes show protein bands at an unexpected size. The expressed protein is slightly larger than GST. This puzzling result gave reason to analyze pRS424-*CnMIP1*#18 with more scrutiny. Sequencing data revealed that a 131 bp portion of unwanted DNA had been

ligated into the plasmid between the GST tag and *CnMIP1*. This extra DNA caused a frameshift in the translation process and resulted in a truncated recombinant protein.

III. E *Correcting the pRS424-CnMIP1#18*

The pRS424-*CnMIP1*#18 plasmid had to be corrected in order to move forward expressing CnPolG in *S. cerevisiae.* In order to correct this plasmid, the Exon1F primer was used with BLO245 in a PCR reaction to remove the unwanted DNA and the PCR product was ligated to itself using NEBuilder HiFi cloning (see Materials and Methods). The newly synthesized plasmids were confirmed with Sanger sequencing to contain *CnMIP1* in the correct orientation and with no unwanted DNA included.

III. F *Native-CnMIP1 expression.*

Now that pRS424-*CnMIP1* had been obtained, it was pertinent to make sure that the protein would express robustly and that protein products were not degraded as heavily as in bacteria.

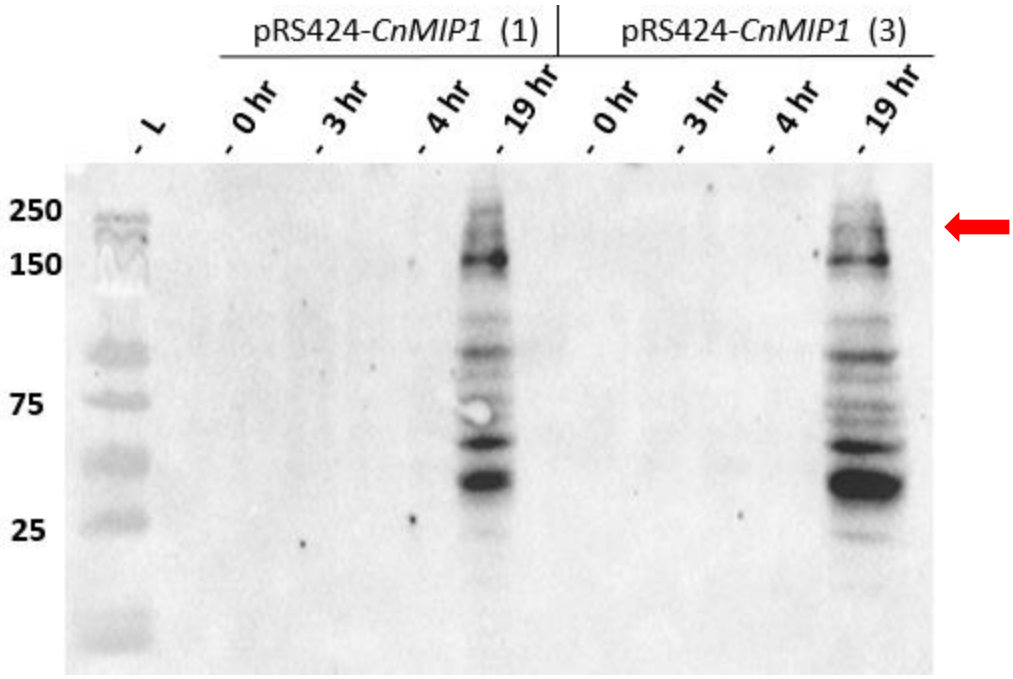

Figure 10. Expression of CnPolG in *S. cerevisiae* with pRS424-*CnMIP1*. Western blot performed with GST antibody. pRS424-*CnMIP1* was the expression vector used. This gel represents the results of induction of two individual transformant colonies 3 hours 4 hours and 19 hours after induction.

Figure 10 revealed that our protein was expressing very slowly. After 4 hours there was no detectable GST-tagged protein in *S.* cerevisiae lysate. After 19 hours, the GST-tagged recombinant protein products were visible, but the presence of multiple lower weight bands indicated that the products were heavily degraded. This concerning result urged the acquisition of an *S. cerevisiae* codon-optimized version of *CnMIP1*.

III. G *Sc-codon-optimized-CnMIP1 expression*

The excessive degredation of protein expressed in Figure 10 led to the acquisition of an *S. cerevisiae* codon-optimized version of *CnMIP1* (*CnMIP1-Sc*). The new DNA fragment was transformed into *S. cerevisia* along with pRS424 linearized with EcoRI and XhoI in order to perform homolgous recombination (see Materials and Methods). Protocols designed to recover

the plasmid from yeast proved unsuccessful with the transformed yeast. However, transformant colonies taken directly from the tranformation plate were capable of expressing recombinant CnPolG for up to five days after transformation.

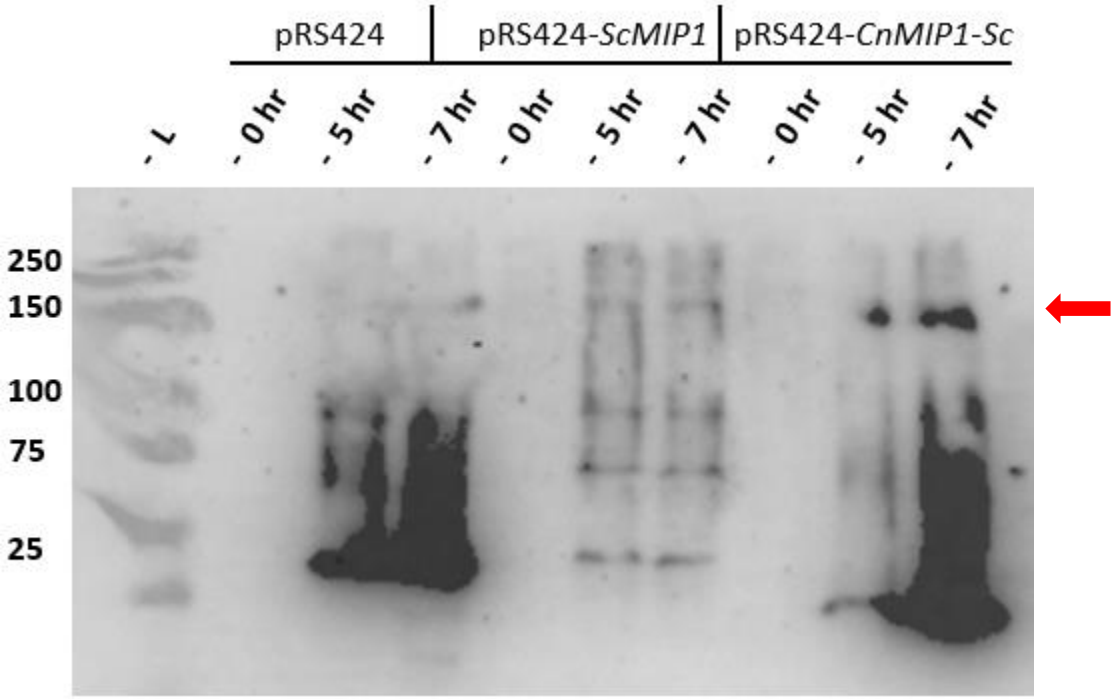

Figure 11. Expression of GST, ScPolG, and codon optimized CnPolG in *S. cerevisiae*. Western blot performed with GST antibody. Protein expressed using pRS424, pRS424-*ScMIP1*, and pRS424-*CnMIP1*#18 are represented. A band at 25 kDa is expected from GST, a band at 160 kDa is expected from ScPolG, and a 183 kDa band is expected from CnPolG.

In Figure 11 the pRS424 lanes showed that GST was still being produced robustly. The pRS424-*ScMIP1* plasmid was producing minorly degraded ScPolG. Finally, the pRS424-*CnMIP1-Sc* lanes showed that the transformant colonies from the homologous recombination plates could produce full-length CnPolG. The 5-hour time point contained less degraded protein than the 7-hour time point, so moving forward 5 hours would be considered the optimal induction time.

III. H *Assessing Sc colonies for protein production*

In order to show consistent ability for the homolgous recombination transformants to produce recombinant CnPolG, 14 different colonies were induced at 30 °C and assessed via western blot.

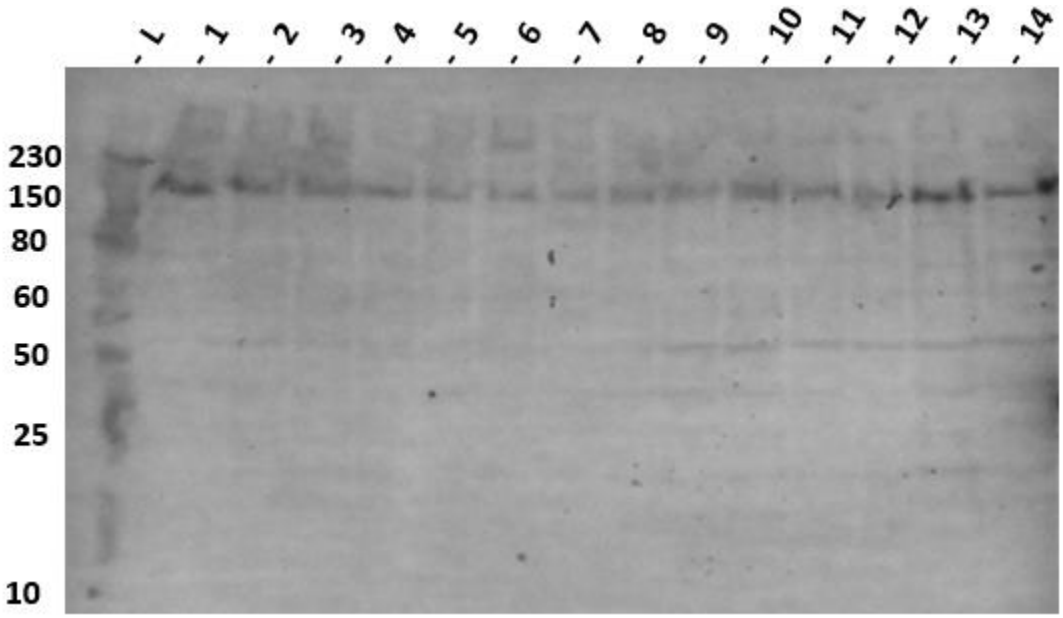

Figure 12. Expression of CnPolG from 14 homologous recombination transformants. Western blot performed with GST antibody. 1-14 indicate 14 different *S. cerevisiae* colonies 5 hours after induction of protein expression with galactose.

The *S. cerevisiae* colonies assessed in Figure 12 showed that homologous recombination was consistently producing colonies capable of inducible protein expression. Though some of the colonies seemed to produce protein more robustly (lane 13), all of these colonies produced protein that was minorly degraded.

Moving forward these colonies were used in larger scale experiments. However, the protein expression phenotype was lost in these large-scale induction. Three to five days after transformation, even small scale inductions lost the ability to produce recombinant protein.

IV. *Characterization of the Polymerase activity of CnPolG using M13 assays*

The M13 assay utilizes circular, single-stranded, viral DNA as a template for DNA polymerization. Results from this assay can be used to confirm polymerase activity of an enzyme, explore optimal buffer components, estimate the velocity of an enzyme's polymerase activity and it can determine whether a polymerase is capable of strand displacement.

IV. A *CnPolG shows DNA polymerase activity*

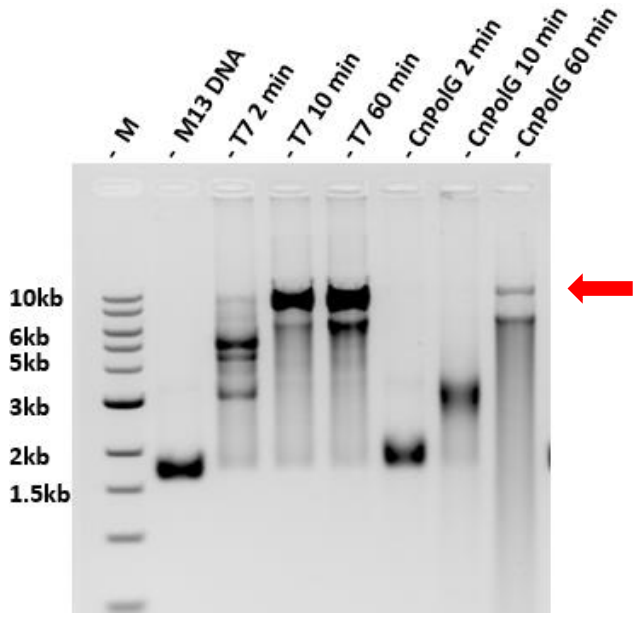

Figure 13. M13 Rolling Circle assay utilizing polymerase and CnPolG. Single-stranded M13 DNA was used as template for both proteins. Products formed at the indicated time points in the presence of the polymerases were visualized on a 0.8% agarose gel.

The assay utilized the recombinant protein containing CnPolG with a N-terminal His_6 tag and a C-terminal Strep-tag® (Figure 13). This double-tagged protein was acquired from Dr. Wallen. Single-stranded M13 DNA annealed with $M13^{26}$ primer was used as the template in these reactions and was represented by a band in the M13 DNA lane between 1.5 kb and 2 kb. Depletion

of template band intensity and appearance of higher molecular weight DNA species indicated successful polymerization of the single-stranded DNA template.

T7 DNA polymerase was used as a control of the polymerization assay. It provides an example of a polymerase that does not perform strand displacement but, polymerizes DNA robustly. As seen in Figure 13, the T7 polymerase protein was able to utilize the M13 template for rapid polymerization, with most of the ssDNA being replicated by 10 minutes. Within an hour (T7 60 min lane) the fully replicated DNA manifested as two bands, at approximately 7 kb and 8 kb. Depletion of the template DNA band further confirmed that this assay was demonstrating efficient DNA polymerization.

Although CnPolG also showed polymerization activity, as indicated by two M13 bands after one hour, it was a much slower enzyme under these conditions in comparison to the T7 polymerase. There was minimal depletion of the ssDNA template after 2 minutes, and partial polymerization after 10 minutes. After 60 minutes there seemed to be a definitive end point for the DNA replication showing that CnPolG was capable of fully replicating the DNA template. It provided evidence that CnPolG does not perform strand displacement. It also showed that something else was occurring which caused a low weight DNA smear smaller than the original template. This could be the result of exonuclease activity.

IV. B *CnPolG shows sensitivity to NaCl and different function depending on cation*

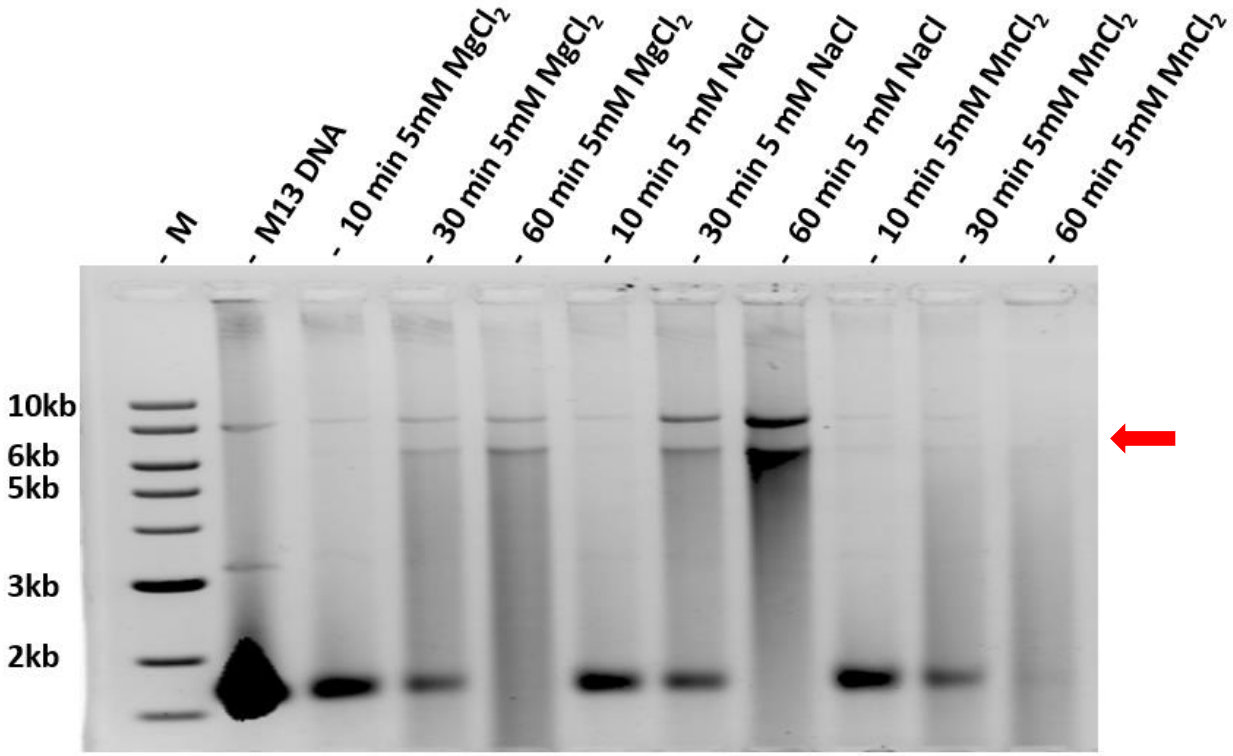

Figure 14. Effect of Mg^{2+}, Mn^{2+} and low NaCl concentration on the CnPolG polymerase activity in the presence of a non-tailed primer. Products of the M13 Rolling Circle assay using His_6-tagged CnPolG were visualized using a 0.8% agarose gel. This assay uses M13 ssDNA annealed with M13[26] primer. The times of incubation, cations, and the salt concentrations used are indicated.

The M13 Assay represented by Figure 14 shows several interesting results. The M13 ssDNA template showed up as a band at the expected size between 1.5 kb and 2 kb. However, the presence of lighter bands near 3 kb and 8 kb suggested that the template may be damaged or contaminated in some way. Presence of these bands in the polymerase reactions, are therefore not necessarily indicative of full-length replication of the ssDNA template.

In order to investigate the effects of salt concentration on CnPolG polymerase activity, the concentration of NaCl was lowered. The standard concentration of sodium chloride in the assay is 20 mM. In the lanes marked as 5 mM NaCl, the concentration was reduced to 5 mM. All

other conditions are the same as the 5 mM $MgCl_2$ lanes. The 5 mM NaCl lanes show the ssDNA template was depleted more rapidly than the 25 mM NaCl concentration lanes. The band at approximately 8 kb in the 60-minute 5 mM NaCl lane was significantly darker than the 60-minute 5 mM $MgCl_2$. This could mean that the CnPolG enzyme prefers a lower NaCl environment.

Another interesting difference between the 60-minute 5 mM NaCl lane and the 60-minute 5 mM $MgCl_2$ lane was that the low weight smear that appeared below the M13 ssDNA template was lighter in the 5 mM NaCl lane. This could mean that whatever was causing this smear may be mitigated by lowering NaCl concentration.

The last three lanes showed the result of performing this assay utilizing Mn^{2+} in place of using Mg^{2+} as a metal cation. This reaction mixture also utilized the standard 25 mM NaCl concentration. The 60-minute 5 mM $MnCl_2$ lane showed that the M13 ssDNA template had been almost completely depleted similarly to the other 60-minute lanes in this gel. However, the appearance of a band at approximately 8 kb was not present in the 5 mM $MnCl_2$ assay. This is puzzling because the low weight smear was also lighter than the other two 60-minute lanes. Since the ssDNA template was thoroughly depleted it is not clear what happened to all the DNA which should have presented itself as bands at a higher molecular weight.

IV. C *CnPolG shows no evidence of strand displacement*

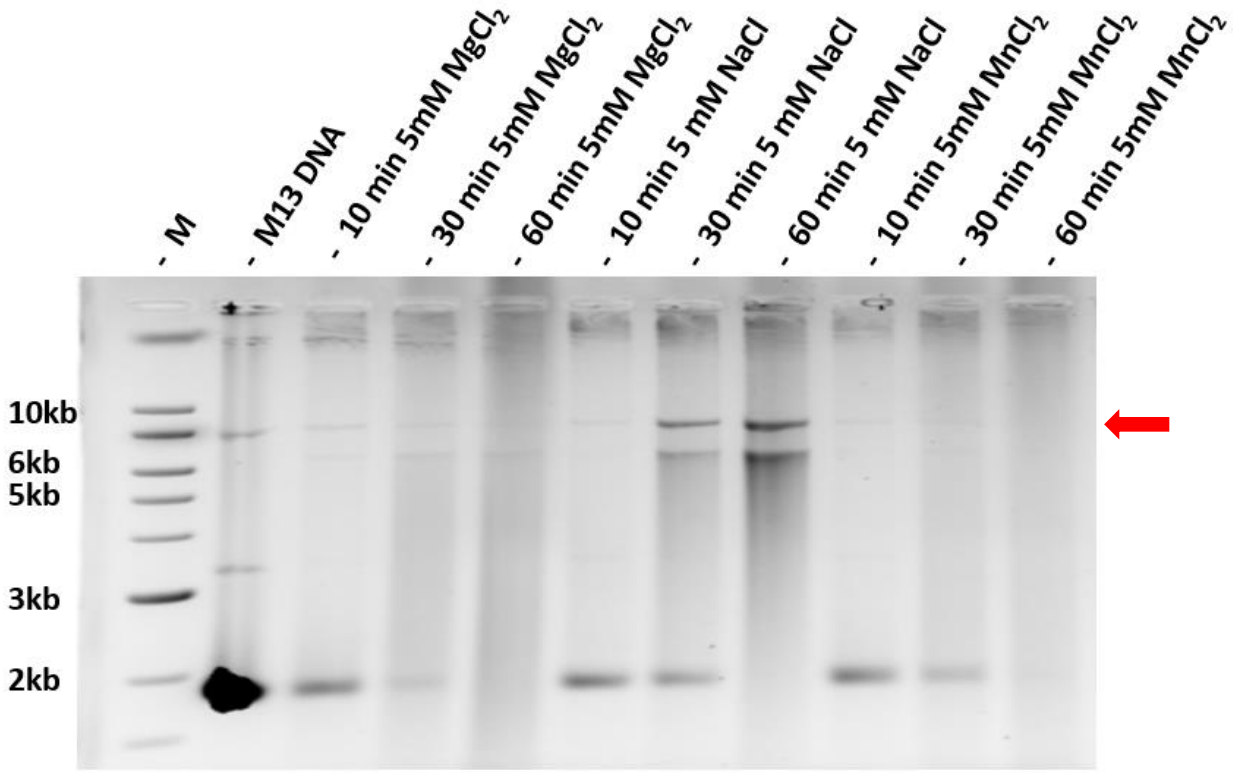

Figure 15. Effect of Mg^{2+}, Mn^{2+} and low NaCl concentration on the CnPolG polymerase activity in the presence of a tailed primer. Products of M13 Rolling Circle assay using protein containing His_6-tagged CnPolG were visualized using a 0.8% agarose gel. These results explore the effect of lowering NaCl concentration in the M13 buffer as well as utilizing $MnCl_2$ as a metal cation in place of $MgCl_2$. This assay uses M13 ssDNA annealed with M13 tailed primer. The times of incubation, cations, and the salt concentrations used are indicated.

The gel in Figure 15 represents the same assay conditions as the gel in Figure 14, but the primer annealed to the ssDNA Template includes a 10 bp non-complementary tail which was designed to increase the chance that the enzyme will perform stand displacement. In this assay it seemed that very similar results were achieved compared to Figure 14, including the two bands present above the ssDNA template. The addition of a non-complementary tail showed no

evidence that strand displacement was occurring when utilizing this enzyme for DNA polymerization.

IV. D *Divalent cations are essential for CnPolG function*

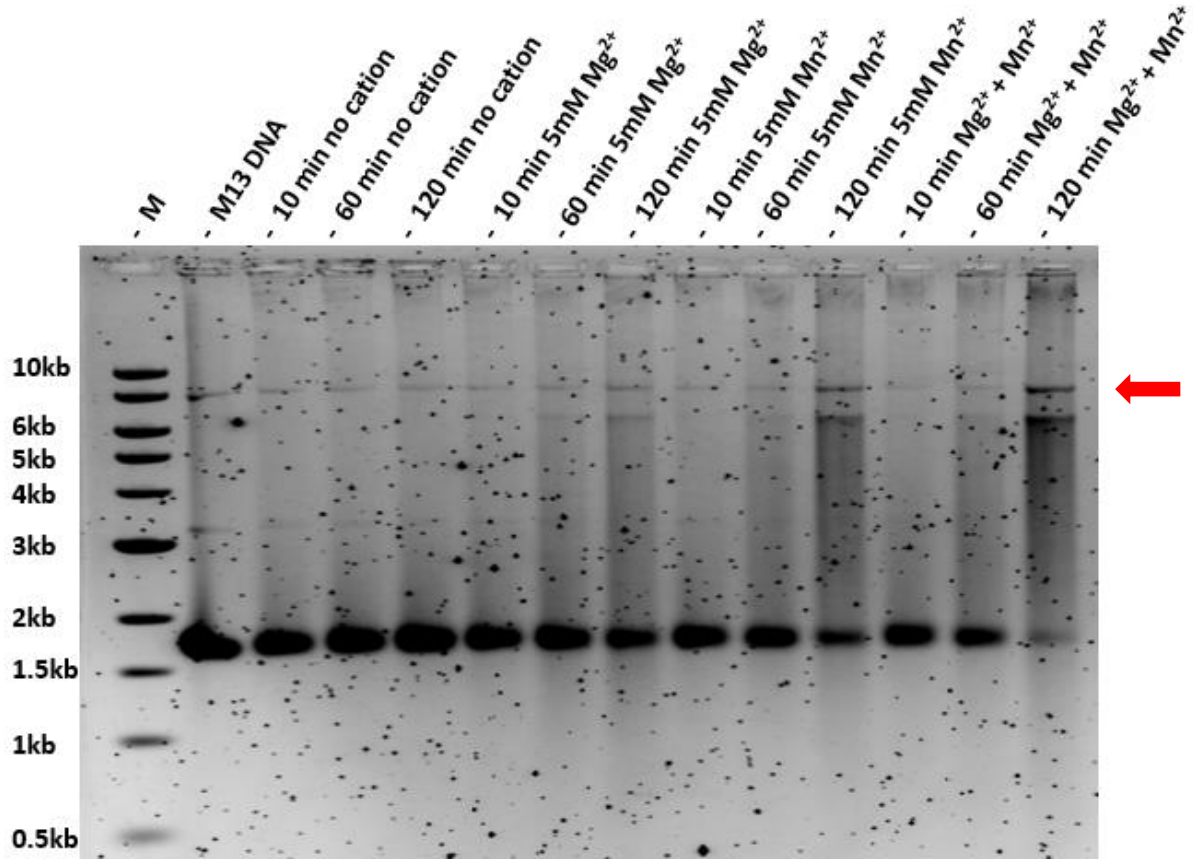

Figure 16. Effect of cations on the CnPolG polymerase activity. Products of M13 Rolling Circle assay using His_6-tagged CnPolG were visualized using a 0.8% agarose gel. The times of incubation, cations, and the salt concentrations used are indicated. This assay uses M13 ssDNA annealed with $M13^{26}$ primer.

Figure 16 shows the results of an M13 assay used to display the essentiality of metal cations to the polymerase activity of CnPolG. The M13 DNA only lane contained the same two light bands above the expected size. Therefore, the light, roughly 8 kb band in the no metal cation lanes, can be explained by the presence of that same band in the DNA template. The no cation lanes also showed negligible depletion of the template DNA, and showed no band

appearing at roughly 7 kb (as in the bands which have shown fully polymerized DNA). Together this provided ample evidence that CnPolG was unable to polymerize DNA without a cation.

The 5 mM Mg^{2+} lanes showed that the addition of magnesium cations was sufficient for enabling DNA polymerization. Although in this assay it seemed that much of the DNA template remained unreplicated. The lanes containing 5 mM Mn^{2+} showed a similar result to the magnesium lanes but appeared to have used up more of the template DNA and produced slightly more fully replicated DNA. The difference between the results shown in Figure 16 here and the results shown in Figures 14 and 15 may be explained by the lower (5 mM) salt concentration.

The Mg^{2+} + Mn^{2+} lanes show that the addition of 5 mM magnesium and 5 mM manganese in the same reaction significantly increased template usage and fully replicated template DNA. This could be due to minor differences between these cations that allow the protein to work more efficiently when used together. This could also reflect the higher concentration of divalent metal cations. Both 5 mM and 10 mM concentrations were in extreme excess to the concentration of the enzyme, so the difference in concentration may not be the reason for the increased reaction velocity.

Chapter IV. DISCUSSION

A. *The function of DNA polymerase gamma is essential in C. deneoformans*

C. neoformans/C.deneoformans (*C. neoformans* Serotype A and D that have now been classified as separate species) are obligate aerobes and therefore cannot survive without mitochondria. In the case of a facultative anaerobe like *S. cerevisiae*, loss of this gene leads to complete loss of mitochondria,[24] so it is likely that this gene is in fact essential in a strict aerobe like *C. neoformans/C. deneoformans* as well. In order to determine this, the gene was knocked down by RNAi. The opposing *GAL7* promoters were simultaneously induced in the presence of galactose, leading to the formation of two complementary mRNA sequences that ultimately form dsRNA. The Dicer enzyme then cuts the mRNAs into small interfering RNAs (siRNA). RNA-induced silencing complex (RISC) uses the siRNAs to locate and degrade all mRNAs with complementary sequences. Expression of RNAi via the pIBB103 plasmid is variable in *C. deneoformans* cells, possibly because the plasmid is not maintained via a cryptococcal autonomous replication sequence (ARS), and is probably prone to plasmid loss. That is why the presence of the *ura5* fragment helps in assessing viability of the second gene.

The tandem silencing of *URA5* provides a negative selection factor to confirm efficient RNAi by utilizing 5-FOA. 5-FOA has no effect on strains of *C. neoformans* deficient of *URA5* because the *URA5* protein product, orotidine 5'- phosphate decarboxylase, normally utilizes orotic acid to produce uracil. It is also capable of utilizing 5-FOA which produces a toxic compound known as 5-flourouracil. The RNAi experiments were performed in *C. deneoformans*

since this assay does not work as well in *C. neoformans*, presumably because the *GAL7* promoters in pIBB103 are from the former species.[42]

More evidence that *CnMIP1* is essential can be provided by replacing the native genomic promoter of this gene with the copper regulatable promoter, *CTR4*. This promoter has been shown to mediate high levels of expression in the absence of copper and tightly repress gene expression in the presence of copper.[42] Replacing the native *CnMIP1* promoter with the *CTR4* promoter could potentially be performed with relative ease using the CRIPSR/CAS9 system.[43,44] If using this molecular switch to turn off *CnMIP1* expression proves fatal, it will provide great evidence of essentiality of this gene.

B. *Expression of CnPolG in bacteria is a viable means of CnPolG expression*

E. coli is commonly used as a first choice for expression of recombinant proteins due to the ease of the system and the low cost. Therefore, initially the CnPolG protein was expressed in this system using the pET28a-*CnMIP1* plasmid gifted by the Wallen lab. This plasmid utilizes a kanamycin resistance gene as a selection factor and carries an in-frame N-terminal His_6-tag so that the expressed protein may be purified using a Ni-column. However, induction of the CnPolG protein at the standard temperature of 37˚C showed extensive degradation. In order to try and purify the full-length protein, the eluants from the Ni-column were passed over an Ion-exchange column. Ion-exchange chromatography works by loading the impure protein sample on to the column at a particular pH. Charges on the proteins bind opposite charges present in the column's resin. Full-length CnPolG is presumed to have a different charge than the degraded products. Proteins with less exposed charged functional groups should elute from the column at low concentrations of NaCl compared to those with many exposed, charged functional groups. Therefore, incrementally increasing NaCl concentration in the elution buffer allows segregation

of the proteins. This treatment did lead to elimination of many of the smaller fragments, but the yield of full-length protein was still low. CnPolG is a large protein of 1421 amino acids, and with the His_6 tag has a predicted mass of 158.7 kDa. It is possible that the large size of the protein leads to steric strain inside the *E. coli* cells, or the protein has some sort of negative effect on the cells. Since this protein has shown a potential tendency to degrade DNA as observed by the appearance of a DNA smear below the size of the DNA template (Figure 13), it could be that this protein is degrading genomic *E. coli* DNA causing the bacteria to target this protein for degradation.

Cryptococcal proteins may also need specific conditions of pH or temperature in order to fold correctly. Improper folding of this protein upon translation could also be increasing degradation[45] and might be mitigated by co-expression of *C. neoformans* chaperones or by moving to a more closely related species to *Cryptococcus*.

In order to try and mitigate the degradation, the Wallen lab had already tried tagging this protein at both ends, with a Strep-tag at the C-terminus as well as a N-terminal His_6. Even after scaling the experiment to 27 liter of growth media, yields of full-length protein were too low to justify the amount of supplies and effort this procedure demands.

Growing *E. coli* in the presence of ethanol before induction of the protein with IPTG following the previously mention protocol[39] has shown promising results. Ethanol is an amphipathic molecule. When it is exposed to bacterial cell membranes, it causes changes in membrane fluidity, membrane transport and membrane lipid composition.[40] This causes an increase in DNA synthesis[41] which could contribute to increased protein expression levels in the presence of ethanol. If the level of increase is high enough, this method could justify utilizing pRS424-*CnMIP*-His-Strep again to express protein in the presence of ethanol.

C. *Expression of CnPolG in yeast was clean but unstable*

Considering the troubles encountered when expressing recombinant CnPolG in bacteria, moving to a different organism for protein expression seemed like a logical option. CnPolG is native to a eukaryotic yeast which utilizes a very different arsenal of cellular machinery to translate mRNA to protein. *S. cerevisiae* is also a eukaryotic yeast and methods of expressing recombinant protein have been well established in this organism.[46]

A modified pRS424 plasmid was chosen as the expression vector for yeast expression. This plasmid uses *TRP1* to allow for growth on -TRP. It contains bacterial and yeast origins of replication to allow for rapid modifications using bacteria. The plasmid is also equipped with a *GAL1* promoter to allow for expression of recombinant protein. *GST* followed by an HRV-3C site precede the multiple cloning site allowing for purification of the GST-tagged protein and removal of the GST-tag with HRV-3C protease. In this case the cloned *CnMIP1* gene was oriented to tag the N-terminal end of CnPolG with GST.

Due to the inability of *S. cerevisiae* to efficiently splice introns from genomic DNA, only the coding sequence could be cloned into the pRS424 plasmid. The first method attempted was to use HiFi cloning which utilizes exonuclease, ligase and proofreading polymerase to piece together overlapping PCR products. *CnMIP1* consists of four exons, so PCR products were generated of each exon with overlaps of their adjacent exons. The first and fourth exons contained overlaps with pRS424. Multiple attempts failed to generate the desired plasmid.

The plan was to use cDNA as a template for PCR which led to the results shown in Figures 6a and 6b. *CnMIP1* coding sequence was then cloned in pTOPO with N-terminal XhoI and C-terminal EcoRI restriction sites. When cut with both restriction enzymes, ligation of the

gene into pRS424 was never successful. No plasmids recovered from post-ligation transformation showed any evidence of containing *CnMIP1*. Finally, pTOPO-*CnMIP1* digested with EcoRI alone was ligated into pRS424. Twenty transformant colonies from this ligation were screened and only one of them contained the gene in the correct orientation. However, when used to express protein, this plasmid was producing a protein product far smaller than the expected molecular weight (Figure 9). Upon inspection by Sanger sequencing, it was revealed that this plasmid contained a small fragment from pTOPO and the mitochondrial targeting sequence which was intended to be left out. Correcting this plasmid (pRS424-*CnMIP1*#18) consisted of creating a primer that was an antiparallel copy of Exon1F, using these primers to produce a PCR product with overlapping ends, and using HiFi to ligate the ends together. All these steps required multiple attempts. This finally resulted in pRS424-*CnMIP1*.

Proper expression of the CnPolG was verified by western blot with GST antibody. Protein expressed with pRS424-*CnMIP1* should result in a protein product of 188 kDa. The result of expression with this plasmid is shown in Figure 10. This could be explained by the long induction period, but undetectable levels of protein were being expressed in lesser amounts of time. Codon optimization of heterologous genes is a common tool that can lead to improvement of protein translation.[47] The company GenScript was tasked with codon optimizing *CnMIP1* to *S. cerevisiae* and cloning the codon optimized gene in pRS424. GenScript successfully codon optimized this gene and stored it in pFL39. However, all attempts made by this company to clone the gene into pRS424 failed. Attempts by the Wallen lab and Bose lab to produce a pRS424 plasmid containing *S. cerevisiae* codon optimized *CnMIP1* also failed. Transforming yeast with PCR products of BLO256 and BLO257 and pFL39-*CnMIP1*-Sc and pRS424 digested with XhoI and EcoRI seemed to be sufficient for homologous recombination, and protein expression was

verified by western blot using these transformant colonies (Figure 11). Attempts to recover plasmids from these colonies failed, and interestingly, after 3-5 days these colonies lost the protein expression phenotype.

D. *CnPolG is a polymerase that prefers low salt concentrations*

CnPolG is capable of robust polymerase activity. Interestingly, the recommended NaCl concentration of 20 mM seems to cause CnPolG to function more slowly. DNA smearing below the M13 ssDNA template also seems to increase in the 100 mM salt lanes. Human PolGA has been shown to reduce polymerase activity and increase exonuclease activity in the presence of high salt concentration.[30] It could be that the low DNA smear is the result of the activation of the exonuclease domain in high NaCl concentration. When human PolGB is used with human PolGA, the salt tolerance of human PolGA increases. This could mean that CnPolG has a processivity cofactor similarly to human PolGA.

E. *CnPolG tagged with His_6 and Strep outperforms protein tagged only with His_6*

The single tagged CnPolG seems to polymerize DNA at a slower rate than the double tagged protein when the same conditions are used. This can be inferred because 10 minutes after the reaction was started, the DNA band is still at the same molecular weight as the template ssDNA using the single tagged protein (Figure 14), but at the 10 minute mark using the double tagged protein there is evidence that the M13 ssDNA is partially replicated (Figure 13). This may be due to some degraded protein products present in the final protein solution. It is possible that some of the degraded CnPolG is still capable of binding DNA without being able to polymerize that DNA. This would most likely slow down the reaction or prevent it in the first place. Seeing

that much of the template remains undepleted in these reactions, it would make sense that something is preventing the template from being used entirely.

F. *Mn^{2+} and Mg^{2+} effect CnPolG differently*

This study has shown that CnPolG is incapable of DNA polymerization without the help of a divalent cation such as Mn^{2+} or Mg^{2+}. This result was expected, as Family A polymerases require these cations to function. What is interesting about CnPolG is that it seems to utilize the M13 ssDNA template differently depending on which cation it has to work with. It has been previously shown that Mg^{2+} cations make human PolGA prefer to use DNA substrates while Mn^{2+} cations make it prefer RNA substrates.[30] However, in these experiments it seems that the use of Mg^{2+} caused more fully replicated M13 DNA to appear while using up all the template differently than Mn^{2+} which used up the template but did not produce fully replicated DNA. This could be because the polymerase domain is activated more in the presence of Mg^{2+}.

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APPENDIX A

A. Primers:

Table 1. Primers used in this project

Name	Description	Sequence (5' to 3')	Restriction Enzyme
BLO001	*CnActin* (F)	CTTGGTCATTGACAATGGCTC	NA
BLO002	*CnActin* (R)	CTTGGTCATTGACAATGGCTC	NA
BLO179	*CnMIP1* localization F	GACAACGACTTCACCAATCCATGCG CAAGGCGCTTGATATTTC	NA
BLO180	*CnMIP1* localization R	CTCGCCCTTGCTCACCATTGCTTGAA ACTTCTTGCTAGAAGACAGAAC	NA
Exon1F	Exon1-pRS424 overlap	AGTTCTGTTCCAGGGGCCCGAATTC ATGAAACCATCGGATGCACC	EcoRI
Exon1R	Exon1-exon2 overlap	CGAATCTTCCTAACTCGAGGCTGGC CATAGC	NA
Exon2F	Exon2-exon1 overlap	GGCCAGCCTCGAGTTAGGAAGATTC GCAAATCCAAATTGC	NA
Exon2R	Exon2-exon3 overlap	TTCCGCCGCGGGCCGCTCTTGCTTTG CCCCG	NA
Exon3F	Exon3-exon2 overlap	CAAAGCAAGAGCGGCCCGCGGCGG AATCCTG	NA
Exon3R	Exon3-exon4 overlap	GATGAGGTGATATCTTGAGTTGTGG GTGTCCAGTCAAGC	NA
Exon4F	Exon4-exon3 overlap	CCCACAACTCAAGATATCACCTCAT CCAATGATG	NA
Exon4R	Exon4-pRS424 overlap	TAGCTTGGCCGGGCCCCCCCTCGAG TCATTGAAACTTCTTGCTAG	XhoI
BLO245	Exon1F Antiparallel	GGTGCATCCGATGGTTTCATGAATT CGGGCCCCTGGAACAGAACT	EcoRI
BLO246	*CnMIP1ΔN*	TTCTGTTCCAGGGGCCCGAATTCAT GCGGAATCCTGTTGGGGTCCAAAT	XhoI
BLO247	*CnMIP1ΔN*	ATTTGGACCCCAACAGGATTCCGCA TGAATTCGGGCCCCTGGAACAGAA	EcoRI
BLO248	pRS424-PIF1 HiFi F	AGTTCTGTTCCAGGGGCCCAAGCCT ATGCCAAAACCAGGTTTCTACG	EcoRI
BLO249	pRS424-PIF1 HiFi R	TAGCTTGGCCGGGCCCCCCCTCGAG CTAATATAATATACCAAAGTTA	XhoI
BLO250	pRS424-PIF2 HiFi F	AGTTCTGTTCCAGGGGCCCAAGCCT ATGCCTATTCTTACGGCCCGCACACT C	EcoRI

BLO251	pRS424-PIF2 HiFi R	TAGCTTGGCCGGGCCCCCCCTCGAG TCATACGAGATTCAAATCCTTGA	XhoI
BLO252	pRS424-*CnMIP1* R EcorI	CTTCTTGAATTCTCATTGAAACTTCT TGCTAG	EcoRI
BLO253	Cn*MIP1*-Sc F	CACAACGAATTCATGAAACCATCAG ATGCTC	EcoRI
BLO254	Cn*MIP1*-Sc R	CACAACGAATTCTTGGAATTTCTTA GAAGAAGAC	EcoRI
BLO255	pRS424-NΔ-*CnMIP1*-Sc HR F	CCGAATTCGATATCAAGCTTATCGA TACCGTCGACCAGAAATCCAGTTGG TGTTC	NA
BLO256	pRS424-NΔ-*CnMIP*-Sc HR R	GCCCGGAATTAGCTTGGCCGGGCCC CCCCTCGATTATTGGAATTTCTTAGA AGAA	XhoI
BLO257	pRS424-NΔ-*CnMIP1*-Sc HR F	ATCAAGCTTATCGATACCGTCGACC GTTAACAGAAATCCAGTTGGTGTTC	EcoRI
BLO273	pRS424-insert Screen F	TTGGATATTAGATACGGTGTTTCG	NA
BLO274	pRS424-insert Screen R	GTGAACCATCACCCTAATCAAGTT	NA
BLO275	M13[26]	CGCGTACTATGGTTGCTTTGACGAG C	NA
BLO276	M13 tailed	CCGCGACCATCGCGTACTATGGTTG CTTTGACGAGC	NA

B. Fungal Strains

Table 2. Fungal strains used in this project

IB#	Strain name	Species	Mating Type	Description (genotypes etc)	Source
IBCN02	KN99	*C. neoformans*	MAT alpha	WT	Heitman lab
IBCN43	JEC21	*C. deneoformans*	MAT alpha	WT (alias 4659)	Doering lab
IBCN64	cap59	*C. neoformans*	MAT alpha	HYG (alias TDY875)	Lodge lab
IBF42	FM113	*S. cerevisiae*	MAT alpha	*ura3–52, trp1–289, leu2–3, 112, prb1–1122, prc1–407, pep4-3*	Bose lab

C. Bacterial Strains

Table 3. Bacterial strains used in this project

E. coli Strain	Description	Source
NEB 5-alpha	fhuA2 a(argF-lacZ) U169 phoA glnV44 a80a(lacZ) M15 gyrA96	New England Biolabs
BL21(DE3)	F-*ompT hsd*SB (rB-mB-) *gal dcm* (DE3)	New England Biolabs
XL-1 blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [*F´ *proAB lacIqZΔM15* T*n10 (*T*etr)]*	Wallen lab

D. Plasmids

Table 4. Plasmids used in this project

Plasmid Name	Description	Markers	Source
pIBB103	RNAi plasmid (*URA5* knockdown)	Amp/NEO^{G418}	Bose lab
pIBB103-*MIP1i*	RNAi plasmid (*CnMIP1*/*URA5* Knockdown)	Amp/NEO^{G418}	Boggs thesis
pET28a-*CnMIP1*	CnPolG bacterial expression plasmid (N-terminal His_6)	Kan	Wallen lab
pET28a-*CnMIP1*-His-Strep	CnPolG bacterial expression plasmid (N-terminal His_6/C-terminal Strep)	Kan	Wallen lab
pTOPO	Gene storage plasmid	Kan	ThermoFisher
pTOPO-*CnMIP1*	Gene storage plasmid with *CnMIP1*	Kan	This work
pRS424	Yeast protein overexpression plasmid (N-terminal GST)	Amp/*TRP1*	Wallen lab
pRS424-*ScMIP1*	ScPolG yeast expression plasmid (N-terminal GST)	Amp/*TRP1*	Wallen lab
pRS424-*CnMIP1*#18	CnPolG yeast expression plasmid containing excess undesired DNA sequence (N-terminal GST)	Amp/*TRP1*	This work
pRS424-*CnMIP1*	CnPolG yeast expression plasmid (N-terminal GST)	Amp/*TRP1*	This work
pFL39-*CnMIP1*-Sc	CnPolG yeast expression plasmid codon optimized for use in *S. cerevisiae* (N-terminal GST)	Amp/*TRP1*	Wallen lab

E. Media Recipes

Table 5. Recipe for Lysogen Broth, Miller (LB)

Component	g/liter
Tryptone	10
Yeast Extract	5
Sodium Chloride	5

Table 6. Recipe for Yeast Peptone Dextrose media (YPD)

Component	g/liter
Tryptone	20
Yeast Extract	10
Dextrose	20

Table 7. Recipe for Synthetic complete minus tryptophan (-TRP)

Component	g/liter
Yeast Nitrogen Base Without Amino Acids	1.92
Synthetic Dropout Mix Minus Tryptophan	6.7
Glucose	5

Table 8. Recipe for SCGL (pH 5-6)

Component	g or ml /liter
Yeast Nitrogen Base Without Amino Acids	1.92 g/L
Synthetic Dropout Mix Minus Tryptophan	6.7 g/L
Glucose	1 g/L
Glycerol	20 ml/L
Lactic Acid	5 l/L

Table 9. Recipe for YPGL (pH 5-6)

Component	g or ml /liter
Yeast Extract	10g/L
Peptone	20 g/L
Glucose	2 g/L
Glycerol	30 ml/L
Lactic Acid	5 l/L

Table 10. Recipe for 5-FOA plates solution 1

Solution I Components	Unit ingredient
Glucose	2 g
Bacto agar	2 g
Uranine and adenine dropout mix	87 mg
10M NaOH	10 µl
Di H_2O	50 ml

Table 11. Recipe for 5-FOA plates solution 2

Solution II Components	Unit ingredient
Yeast Nitrogen Base	10 ml
5 – Flouro Orotic Acid	0.1 g
100x uracil	600 µl
1x adenine	1 ml

Solution I. is autoclaved for 20 minutes and allowed to cool to ~80 °C. Solution II is filter sterilized using a vacuum filter sterilization system. The two solutions are mixed and poured into sterile 15 ml petri dishes and allowed to solidify in the absence of light. The plates are stored in the absence of light.