Drew University
College of Liberal Arts

Characterizing eEF3 Through The study of its Functional Conservation between *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*

A Thesis in Biochemistry and Molecular Biology

By

Kia Bourdot

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Bachelor in Arts
with Specialized Honors in
Biochemistry and Molecular Biology

May 2015

ACKNOWLEDGEMENTS

I am thankful for the hard work of all of my thesis committee members. Dr. Summer Harrison, thank you for taking the time to assist in revisions of this thesis. Dr. Joanna Miller, thank you for refining my microbiological laboratory techniques and scientific writing style. Dr. Sandra Keyser, thank you for helping throughout the process and for sparking my interest in laboratory research in the first place!

I am extremely grateful for the valuable guidance and mentorship of my research advisor, Dr. Stephen Dunaway. Your confidence in me from the start has kept me motivated and on track to achieving my career goals.

A special thank you also goes out to Valentina DelSignore, an invaluable member of the Dunaway lab who has been extremely helpful throughout this project. Thanks, also, to Stephen Silva, who has helped along the way, and to Sarah Colmer, my inspirational predecessor whose undergraduate research made mine possible.

This project would not have been possible without the financial support of the Drew Summer Science Institute, the Beta Beta Beta National Biological Honors Society, Novartis Pharmaceuticals, and the Howard Hughes Medical Institute.

And finally, a huge thanks to all my friends and family who have supported me along this journey.

ABSTRACT

The incidence of fungal infections has increased drastically throughout the past several decades as a result of changes in healthcare systems (Enoch et al. 2006; Martin et al. 2003; McNeil et al. 2001; Mean et al. 2008; Pfaller and Diekema 2010). In order to combat this increased prevalence, researchers must find a novel way to treat fungal infections with high efficacy. Among the many techniques being investigated is the possibility of pharmaceutically targeting unique sequences of the fungal genome. Fungal organisms have a translational protein, eukaryotic elongation factor 3 (eEF3), which is essential for fungal cell viability yet completely absent from mammalian genomes (Andersen et al. 2004; Dasmahapatra and Chakraburtty 1981; Qin et al. 1990). This makes it a promising therapeutic drug target for treating mycoses in mammalian species.

This project sought to determine the degree to which the eEF3 protein is functionally conserved among the lower eukaryotic species that require it for viability. Analysis of eEF3 was conducted by cloning the gene sequence from *Chlamydomonas reinhardtii* (*C. reinhardtii*), a unicellular species of green algae that is a non-fungal lower eukaryote, into *Saccharomyces cerevisiae* (*S. cerevisiae*), a well-characterized model organism for studying fungi and lower eukaryotes. These experiments provide insight on the conservation of the protein between species that are more taxonomically divergent than those that have been studied in the past. Results showed that eEF3 is not sufficiently conserved to allow functional complementation between these two species. This may be a result of structural or functional changes between the eEF3 proteins of these two organisms, or it may be the result of alternate codon usage between the two species.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

5-fluoroorotic acid – 5-FOA
ABC cassettes – ATP binding cassettes
AmpR – ampicillin-resistance
BRE – TFIIB recognition element
C. reinhardtii – Chlamydomonas reinhardtii
CIP – calf intestinal phosphatase
CrEF3 – *C. reinhardtii* eEF3
ddH_2O – distilled, deionoized water
DHPS – dihydropteroate synthase
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DPE – downstream promoter element
E. coli – Escherichia coli
eEF1α – eukaryotic elongation factor 1α
eEF2 – eukaryotic elongation factor 2
eEF3 – eukaryotic elongation factor 3
eIF1 – eukaryotic initiation factor 1
eIF2 – eukaryotic initiation factor 2
eIF5B – eukaryotic initiation factor 5B
eIF6 – eukaryotic initiation factor GTPase
Ex-ScEF3 – exogenous plasmid containing ScEF3
HE-CrEF3 – high expression plasmid containing CrEF3
H.I.V. – human immunodeficiency virus
LB-amp – LB-ampicillin
LE-CrEF3 – low expression plasmid containing CrEF3
NICU – neonatal intensive care unit
Pol II – RNA polymerase II
RNA – ribonucleic acid
rpm – rotations per minute

S. cerevisiae – *Saccharomyces cerevisiae*

S. pombe – *Schizosaccharomyces pombe*

ScEF3 – *S. cerevisiae* eEF3

SpEF3 – *S. pombe* eEF3

ssDNA – single-stranded DNA

TBP – TATA-binding protein

TFIIB – transcription factor IIB

tRNA – transfer RNA

w/v – weight per volume

1. INTRODUCTION

1.1 Kingdom Fungi

In the study of living organisms, taxonomists order life forms into separate categories, based on their genetic and morphological qualities. Phylogenic trees are developed to visually track evolutionary divergence from ancestral life forms. The primary method of classifying organisms relies on the presence or the absence of a nucleus within the cells, which separates prokaryotes from eukaryotes. These two labels stem from their Latin roots, where "pro-" means "prior to," "eu-" means "true" and "karyo" means "kernel," in reference to the nucleus within the cell (Fancher 2004). Prokaryotes lack a nucleus, and they are believed to have originated earlier than eukaryotes. Eukaryotes have organelles within their cells, including a nucleus where their genes are housed. Generally, prokaryotes have smaller cell diameters, on the order of 1-2 μm, compared to eukaryotic cells, which range from 10-25 μm, on average (Nealson and Conrad 1999). Prokaryotes have rigid cell walls, whereas eukaryotes most commonly have a flexible cell membrane to allow for cellular uptake of larger molecules to obtain nutrients. Prokaryotes have varied metabolic pathways, whereas eukaryotes are generally limited to oxygen respiration and pathways that require organic carbon.

Moving further in the classification process, life is organized into three main domains: bacteria, archaea, and eukarya (Figure 1). These three categories were established by the work of Carl Woese, a twentieth-century biologist who reorganized the tree of life based on molecular characteristics of organisms (Koonin 2014). It is

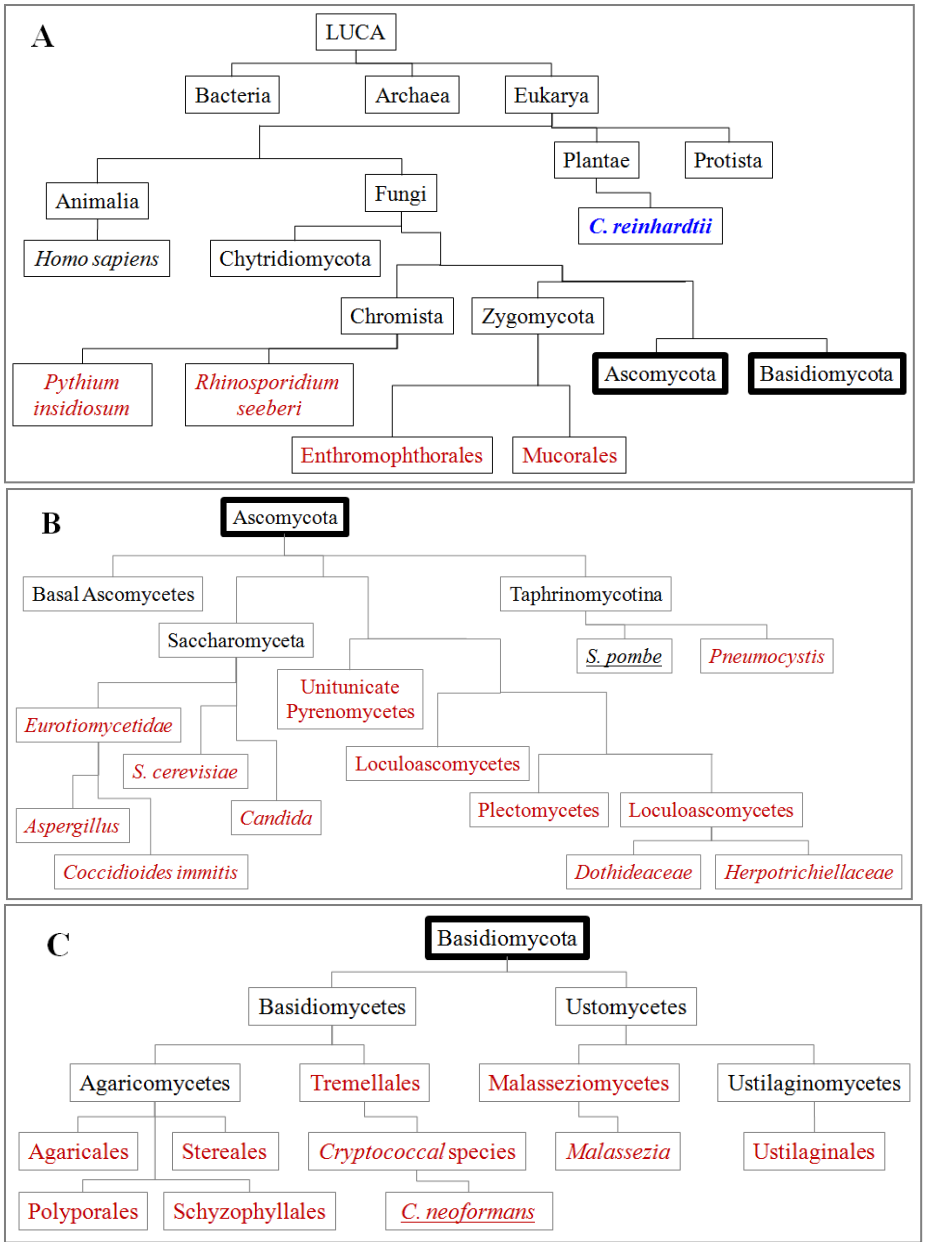

Figure 1 – Phylogenic tree of exemplary pathogenic fungi and of organisms used in this experiment (Guarro 1999; NCBI 2015). In red are the clinically relevant classes of fungi. Underlined species were used in previous experiments to test eEF3 conservation (Blakely et al. 2001; Colmer 2013). Species in blue were used in this experiment. (A) Divergence of fungi from LUCA. (B) Phylogeny of Ascomycota, based on 18S rRNA. (C) Phylogeny of Basidiomycota.

hypothesized that all cells originated from one "last universal common ancestor" (LUCA), which was merely a primitive collection of biological components, not a fully formed cell. From this LUCA, bacteria diverged early from archaea and eukaryotes through a series of genetic mutations. Despite their distanced evolutionary relationship, bacteria and archaea are similar in that they are both prokaryotic, lacking a nucleus and organelles. Bacteria and archaea have smaller cell size than eukaryotic cells, since they lack cellular compartmentalization in organelles. As a result, they typically have smaller genomes than eukaryotes, as well.

It is important to note that even though these three phylogenic domains diverged from each other, they share common qualities of life as a result of horizontal gene transfer and the pressures of evolutionary selection (Ball and Cherry 2001; Koonin 2014). In fact, several organelles present in eukaryotic cells are hypothesized to have originated from the symbiotic relations of bacteria with eukaryotes, which ultimately resulted in the eukaryotic cell engulfing and replicating the bacteria along with its own cells, a process known as endosymbiosis. This explains the evolution of the mitochondria, which is a eukaryotic organelle that contains its own proteome, separate from the cell in which it inhabits (Gray 2012).

The eukaryotic domain includes highly varied species, ranging from single-celled organisms to highly complex life forms that contain systems of cells working together as the tissues, organs, and organ systems that make up one organism. There are four main eukaryotic kingdoms: protista, plantae, fungi, and animalia (Figure 1) (Doolittle 1999). Further protein analysis segregates eukaryotes more specifically into fourteen unique

eukaryotic taxa (Baldauf et al. 2000). These groups are ordered more generally into "lower eukaryotes" and "higher eukaryotes" based on the complexity of their cells and size of their genome.

The fungal kingdom encompasses three main phyla of fungi – Ascomycota, Basidiomycota, and Zygomycota – as well as several smaller phyla that contain fewer organisms (Figure 1). These are identified by differences in their reproductive pathways and physical structures (Guarro 1999). Ascomycota are distinguished by the presence of cylindrical sacs, termed asci, which hold the reproductive spores, and bilayered hyphal walls with an electron differential. Basidiomycota are identified by the presence of basidia, structures that house the spores until the spores reach maturity, at which point the basidia burst to distribute the spores through the air. Yeasts are present in both the phyla Ascomycota and Basidiomycota, but are identifiable at the cellular level; Basidiomycota have scaly inner walls, whereas Ascomycota have smooth walls. Zygomycota are characterized by their coenocytic structure, in which one cell may contain several nuclei and branching hyphal elements, although there may be drastic structural variations between species. There are also deuteromycetes that fall within the gray area between these three main phyla; for this reason, species within this category are sometimes called "imperfect fungi."

Fungal organisms occur ubiquitously around the globe and are able to grow in the soil, in food, in the air, or in or on another host organism (Guarro 1999). Fungi thrive at varied temperature conditions, ranging from -10°C to 61°C (Baxter and Illston 1980; Maheshwari et al. 2000). In fact, they are classified based on the temperatures at which

they grow. Psychrophiles grow optimally when their environment is less than 10°C; mesophiles grow optimally between room temperature and 30°C; and thermophiles grow optimally between 37-50°C (Burge 2006; Maheshwari et al. 2000). These limited temperature ranges within which fungi are able to grow have been attributed to the intrinsic thermostability limits of the proteins present within these eukaryotic cells.

Fungi also grow on and in organisms both alive and dead, driving metabolic reactions of catabolism and fermentation wherever there are adequate organic nutrients (Gottlieb 1963). Fungi are heterotrophs, meaning that they are unable to produce their own nutrients and rely upon their surrounding nutrients for survival. Although fungi are not phototrophic, they are regulated by information relayed by light signaling (Tisch and Schmoll 2010). Most fungi are aerobic, yet some are anaerobic (Gottlieb 1963). In the environment, fungi perform the critical role of decomposing organic materials and nutrients back into their simplest components, an essential recycling role that is critical for other organisms in the environment to flourish. Within plants and animals, fungi thrive as commensal organisms, filling a critical niche of recycling cellular wastes or consumed material that their host organism would not otherwise be able to degrade. Such commensal fungi have co-evolved with their hosts over thousands of years, allowing for the optimization of the benefits of their symbiotic relationship.

1.2 Fungi that Colonize Humans – the Good and the Bad

In humans, there are over eighty genera of fungi that are naturally present within our microbiome (Collins 2013). These commensal organisms help with essential processes of digestion and strengthen natural barriers to infection. Fungi are present on

human skin and within the intestine, adding to the effectiveness of our skin as a barrier to infections and of our digestive system to break down food (Roth and James 1988; Gouba and Drancourt 2015). On the skin, lipophilic fungi of the genus *Malassezia* serve to consume the oils secreted by sebaceous glands, helping to naturally maintain healthy skin conditions to optimize the protective barrier properties of the epithelium (Ro and Dawson 2005; Marcon 1992). Simultaneously, commensal fungi such as *Malassezia* are able to out-compete foreign microbes by utilizing the space and nutrients available, preventing – or at least minimizing – successful colonization by new, possibly pathogenic, fungi and bacteria.

Although fungi perform many important roles, under certain conditions they can run awry, leading to the development of fungal infections, which are known as mycoses in the medical community. Under specific favorable conditions, opportunistic fungi may colonize new hosts or commensal fungi may overgrow within their host, at the expense of the health of their host organism. Such infections may even progress into fatal diseases. Notably, over the past forty years, there has been a significant rise in the incidence of mycoses in the United States and around the globe (McNeil et al. 2001; Martin et al. 2003; Mean et al. 2008). This has largely been the result of increasingly invasive medical procedures, such as grafts, prosthetic surgeries, and indwelling catheterizations, which disturb natural epithelial and mucosal barriers, leaving hosts immunocompromised and susceptible to opportunistic infections that may otherwise have been prevented (Enoch et al. 2006; Pfaller and Diekema 2010). These techniques have led to a rise in cutaneous and subcutaneous infections. The use of immunosuppressive drugs and broad-

spectrum antibiotics also increase the susceptibility of patients to infections by opportunistic microbes such as fungi.

As well as the expanding mechanisms of fungal infection, there are also the endemic pathways of infection that have taken place for thousands of years, which have been minimally impeded by changes in culture and technology. Fungal infections may naturally arise when fungal pathogens overcome the natural cutaneous and mucosal barriers in place that would otherwise defend against infection (Kobayashi 1996; Havlickova et al. 2008). Humans naturally resist fungal infection through the energetic investment of maintaining a relatively high, non-fluctuating body temperature (37°C) through homeostatic regulation measures (Bergman and Casadevall 2010). Such endothermic regulation, typical of warm-blooded animals, has been evolutionarily favored because it limits the ability of most pathogenic organisms to successfully colonize, thereby blocking the first step in the infection process.

However, local fluctuations in environment may allow for the pathogen to colonize. For example, a superficial skin infection may result from a change in hygiene that results in a change in skin pH, moisture, or cellular waste build-up, which may either annihilate commensal microbes or favor conditions for the over-colonization of one or more pathogenic fungal or bacterial species. Even small, physiological changes as a result of a lifestyle change can optimize the ability of a commensal fungal organism to thrive, allowing it to exceed its beneficial presence and accumulate into a pathogenic infection.

An example of commensal fungi turned pathogenic may be seen in *Malassezia*

species that overgrow into chronic diseases in obese, diabetic, or immunocompromised individuals (Marcon and Powell 1992). Organisms in the *Malassezia* genus are normally found in non-pathogenic colonies on human skin near pilosebaceous (oil-secreting) glands, preferentially of the back and chest, and increase in number following puberty, when these oil glands become active. *Malasezzia* have hyphae that aid in the adhesion of the fungal cells to the epithelial cells of humans. These cellular appendages may readily lead to the over-colonization of *Malassezia* species on skin when conditions allow, such as when skin has increased temperature, humidity, or oils. Such skin conditions may result from excess oil or sweat secretion from the skin, which are conditions known as seborrhea or hyperhidrosis, respectively, or from the over-use of moisturizers. Notably, *Malassezia* species have also been known to cause sepsis in the hospital setting when they gain access to the blood stream through catheters.

Clinical cases of *Malassezia* mycoses resulting from over-colonization on the skin present with skin infections marked by pityriasis versicolor (lesions of flaky skin) and folliculitis (periodic lesions that may result in papules or pustules, which may spread rapidly, may result in high fever, and may last up to eight years) (Marcon and Powell 1992). *Malassezia* may colonize in the stratum corneum or in hair follicles, resulting in hyperkeratosis and acanthosis (epithelial inflammation, with possible change in skin pigmentation due to the role *Malassezia* has on blocking melanin production). Topical treatments of selenium sulfide are often prescribed for this type of infection, and such treatments must be applied for a period of up to three months. A high frequency of relapse has been observed in patients, extending this treatment even longer.

Unfortunately, while selenium sulfide is known to act as an anti-fungal agent, it is also a suspected carcinogen for humans (National Toxicology Program 2011). The fact that using this treatment increases cancer risk in patients adds weight to the need for a better anti-fungal treatment.

Natural barriers may also be broken through local wounds, skin-to-skin contact, or sexual contact with an infected individual, greatly increasing susceptibility to mycoses. Inhalation of certain fungal organisms above a particular threshold level may lead to infection if that fungus is able to grow in the lungs or airways, and evade phagocytosis by host macrophages. Fungi may also travel through the bloodstream, paranasal sinus, or ear canal to cause neurological fungal infections (Arunaloke 2007). Individuals with a history of a prior fungal infection are also at risk for a secondary infection resulting from a translocation across a barrier. Secondary mycoses may result from growth across a mucosal barrier, in which the fungal colony accumulates cells that push against and break through to the underlying layers of the skin, from the epithelium to the basement membrane (Pfaller 2010). Secondary mycoses may also result from the passage into the vascular system, which allows the fungal pathogen to be systemically distributed.

Importantly, pathogenic fungi are found in the four major phyla of fungi; this pathogenic quality is not limited to one particular type (Guarro 1999) (Figure 1). The phylum Ascomycota contains the majority (roughly 80%) of the fungal pathogens that cause mycoses, across a broad array of the classes within this phylum. Within the phylum Basidiomycota, most pathogenic fungi are yeasts that fall into the classes Basidiomycetes and Ustomycetes, including *Cryptococcal* species. Among the

Zygomycota, there are two orders containing species that may lead to mycoses: the Entomophthorales and the Mucorales. Additionally, there are two species that cause fungal infections in humans that fall within the much smaller phylum Chromista: *Pythium insidiosum* and *Rhinosporidium seeberi*.

Some well-known examples of invasive fungi include *Candida* species, *Cryptococcal* species, *Aspergillus* species, and *Pneumocystis* species of fungi (Enoch et al. 2006; Pfaller and Diekema 2010). *Candida* infections cause over 80% of hospital-borne fungal infections (Enoch et al. 2006; Jarvis 1995; Jarvis and Martone 1992; Pfaller and Diekema 2010; Rees et al. 1998). They typically arise from endogenous, opportunistic infections as *Candida* species residing on a patient take advantage of their host's immunocompromised state, although *Candida* infections may also arise exogenously through transmission from visitors or healthcare personnel (Asmundsdottir et al. 2008; Bliss et al. 2008; Marco et al. 1999; Pfaller and Diekema 2010). Primarily, these infections are caused by five *Candida* species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. Of these, *C. albicans* is the primary infectious agent. *C. albicans* causes superficial infections of genital, oral, and cutaneous sites, and it also causes bloodstream infections, particularly in stem cell recipients and neonatal intensive care unit (NICU) patients. Infections caused by *C. albicans* are treated with azole antifungals. Symptoms of *C. albicans* infection include neutropenia (an immunocompromised state caused by neutrophil deficiency in the blood) and an underlying solid tumor.

Cryptococcal fungi cause airborne infections, which may be asymptomatic or

result in symptoms including cough, fever, or meningitis (Enoch et al. 2006). Those with human immunodeficiency virus (H.I.V.) and recipients of organ transplants are at increased risk for infection due to their immunocompromised state. Most *Cryptococcal* infections are caused by *C. neoformans*. This disease is spread through the environment via pigeon droppings; pigeons carry the fungal agent to new areas with their beaks and feet (Pfaller and Diekema 2010). Incidence of *Cryptococcal* infections has risen in correlation to the HIV pandemic, yet incidence among populations not infected with HIV has remained steady, indicating that immunodeficiency drastically increases risk of infection (Chayakulkeeree and Perfect 2006; Pfaller and Diekema 2010). If left untreated, infections may progress to cryptococcal meningitis, which has nearly a 100% mortality rate. However, even with treatment, mortality rates remain at about 50%.

Aspergillus infections result from an invasive mold fungus (Enoch et al. 2006). Immunocompromised individuals are at risk, as are patients with blood disorders and H.I.V. autoimmune deficiency syndrome (H.I.V./A.I.D.S.), as well as those undergoing steroid therapy, cytotoxic drug therapy, or organ transplant surgery. Those infected with *Aspergillus* may suffer loss of cough reflexes and a loss of functional cilia along the respiratory tract, leading to chronic congestion within the bronchi of the lungs.

Pneumocystis fungi may cause pulmonary infections (Pfaller and Diekema 2010). This type of infection may result in fatal infections, both in H.I.V.-infected and non-H.I.V. immunocompromised patients (Bin et al. 2006; de Boer et al. 2007; De Castro et al. 2005; Hocker et al. 2005; Manoloff et al. 2003; Morris et al. 2004; Rabodonirina et al. 2004, Roblot et al. 2004). *Pneumocystis* organisms live on warm-blooded mammals.

Airborne infections may be transmitted through reservoirs in rodents, children with or without symptoms, and immunocompromised patients (Pfaller and Diekema 2010; Huang 2011). New evidence also shows that this disease is transmitted between people through surgical transplantation surgeries, particularly for oncology patients (de Boer et al. 2007; Hocker et al. 2005; Manoloff et al. 2003; Rabodonirina et al. 2004; Roblot et al. 2004). *Pneumocystis* has been shown to colonize patients with chronic pulmonary diseases, obstructive pulmonary disease, interstitial lung disease, cystic fibrosis, or lung cancer (Gutierrez et al. 2011). Such complications result from *Pneumocystis* activating alveolar macrophages within their host, thereby triggering damage within their hosts' lungs. *Pneumocystis* is treated using prophylactic sulfonamides. However, this has introduced selective pressures on the pathogen, which has resulted in resistance development through mutations in the targeted protein, dihydropteroate synthase (DHPS) (Pfaller and Diekema 2010).

The toll that fungal infections have can be seen in the number of people who are susceptible to mycoses. Elderly populations are at higher risks due to the increasingly invasive medical care they receive as they combat common diseases of aging (Baddley et al. 2011). Those with chronic illnesses such as H.I.V. are also at high risk due to their immunosuppressed state (Coelho et al. 2014). People who travel to exotic locations or spend a significant amount of time outdoors also increase their risk of exposure to fungal pathogens due to their encounter with new microbes to which they are not biologically accustomed (Baddley et al. 2011). This creates a microbial imbalance that favors the newly abundant pathogens, which pre-existing commensal organisms may not be able to

sufficiently outcompete.

The environment plays a large role in the epidemiology of fungal infections. Some pathogenic fungi are dimorphic, and their life cycles include two different forms. Environmental dimorphic fungi grow in a filamentous form, but following asexual reproduction, they release spores that may infect mammalian species (Pasricha et al. 2013). These spores may be carried to a very different environment, such as a human respiratory tract or digestive tract. When these spores reach host tissues, the fungal organism may undergo a dimorphic switch to grow in its pathogenic yeast form, leading to infection. Infections caused by dimorphic fungi demonstrate seasonal variation in the incidence of new mycoses cases (Baumgardner 2009). This is a result of climatic and ecological factors – including humidity, temperature, soil pH, precipitation levels, wind dispersal, and salinity. For example, cases of coccidioidomycosis, a fungal infection endemic in Arizona caused by *Coccidioides immitis*, spike after there is heavy rain, followed by drought, wind, and high temperatures. Over the past twenty years, the incidence of coccidioidomycosis has increased by over 500%, perhaps due to environmental changes and increased human activity in the area (Baumgardner 2009; Brown et al. 2013). Other dimorphic fungal infections have been on the rise due to changes in weather patterns and human environmental impacts, such as deforestation, pollution, and construction.

Current antifungal compounds include amphotericin B deoxycholate, flucytosine, fluconazole, itraconazole, voriconazole, ravuconazole, posaconazole, anidulafungin, micafungin, and caspofungin (Enoch et al. 2006; Pfaller and Diekema 2010).

Prophylactic combination therapies and are also used (Marr 2004). However, in addition to the increased incidence of fungal infection, these pathogens have also displayed increased resistance to existing therapeutic drugs in use today (Groll and Walsh 2002; Pfaller and Diekema 2010). In recent years, extended-spectrum drugs, such as triazoles and echinocandins, have also been developed to expand the effective treatment of mycoses, but already, fungal pathogens are developing resistance to these newly synthesized drugs (Pfaller and Diekema 2010).

Worse, even if resistance has not yet developed against a particular anti-fungal agent, those on the market today are associated with highly toxic side effects, sometimes resulting in even greater patient discomfort than the infection itself (Enoch et al. 2006; Groll and Walsh 2002; Pfaller and Diekema 2010). Such risks include nephrotoxicity, fevers, chills, nausea, vomiting, hypotension, tissue hypoxia, menstrual disorders, allergies, or sepsis. There are also risks of anti-fungal agents interfering with other drugs that immunocompromised individuals may require. The harm that these treatments cause is compounded further by the fact that most anti-fungal agents require high-dose, long-term treatments, which may last from several months up to several years for recurring infections.

1.3 Molecular Genetics – from DNA to RNA to Functional Protein Machines

What can be done to reverse the rising trend in fungal infections and to offer patients a more reliable treatment of these infections, with high efficacy and low toxicity? In order to tackle that question and determine a potential pharmacological method to target fungal pathogens, it is important to first understand the basic rules governing

cellular life. Researchers make use of their knowledge of these conserved processes to identify unique elements that may be selectively targeted in pathogens in order to create a pharmaceutical compound that may effectively, efficiently, and efficaciously kill such pathogens to restore health. In particular, researchers seek to target unique proteins, since protein structures tend to vary between organisms as the result of differences in their encoding gene sequences and in the post-translational modifications unique to certain organisms, or to particular cell types within an organism.

All biological cells follow the central dogma of molecular biology, a summary of how genotypic information becomes phenotypic expression unique to each cell. Deoxyribonucleic acid (DNA) is transcribed into ribonucleic acid (RNA), which is then translated into amino acid protein sequences (Figure 2). This process is carried out in a universal manner in all living cells. Following translation, the amino acid chains of eukaryotic organisms are folded into functional protein structures through post-translational modification.

Gene transcription in eukaryotic cells involves several proteins in concerted roles that carry out four sequential steps of protein assembly on the template strand, initiation of transcription of DNA to RNA, elongation of the new RNA strand, and termination of the process. Transcription must begin at a promoter sequence along the DNA. This promoter sequence is recognized by RNA polymerase, the enzyme that reads DNA template to produce a complementary RNA transcript. There are three distinct eukaryotic RNA polymerases (Krishnamurthy 2009; Vannini and Cramer 2012). RNA Polymerase I and III transcribe ribosomal RNAs, transfer RNAs, and small nuclear RNAs. RNA

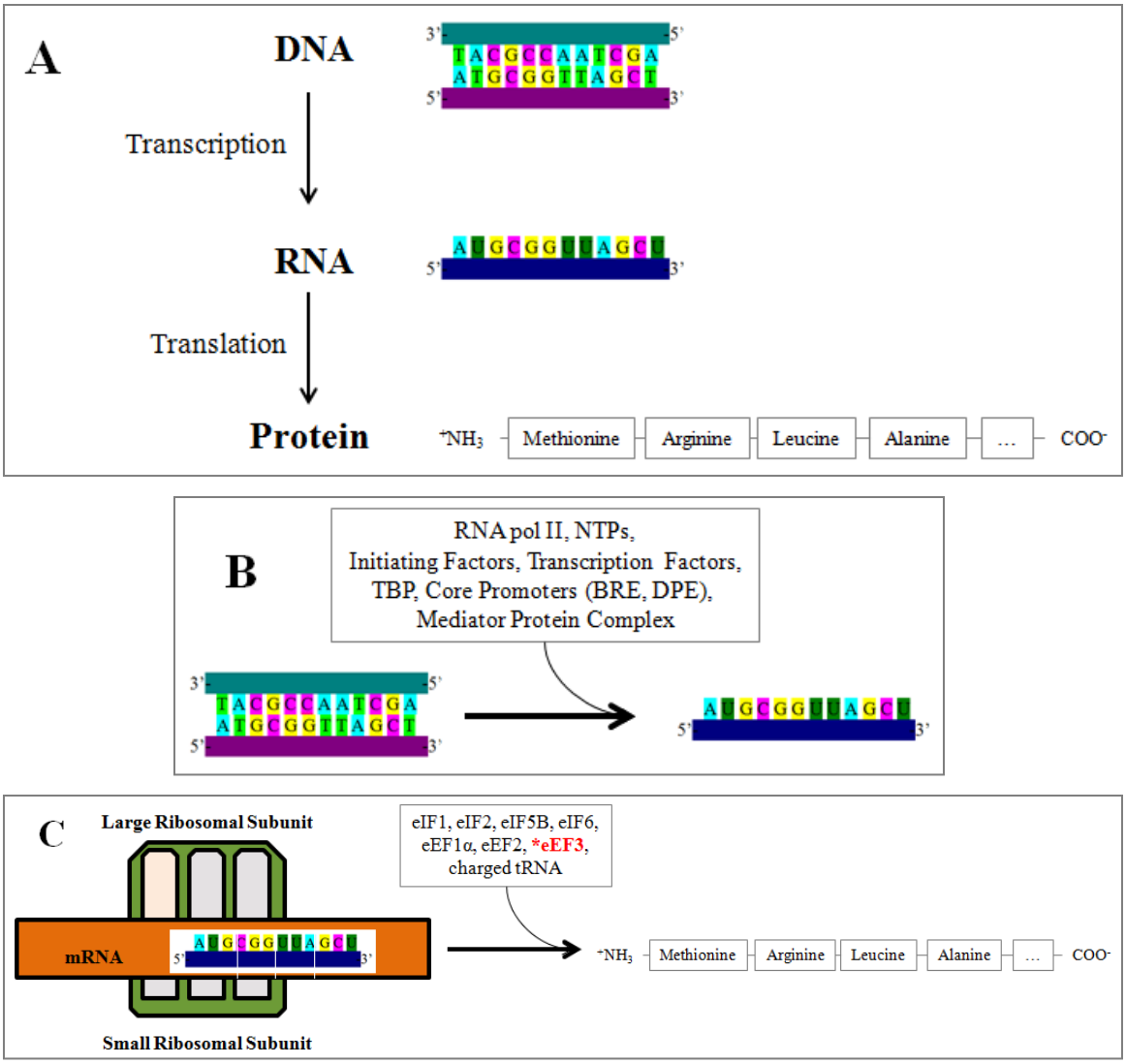

Figure 2 – The central dogma of biology (adapted from Cox 2010). (A) Overview: the encoded genotype of DNA is transcribed into an mRNA construct, which is then translated into a polypeptide protein sequence, which becomes modified post-translationally to yield a particular cellular phenotype. (B) The components involved in transcription. (C) The components involved in eukaryotic translation.

Polymerase II (Pol II) generates messenger RNA (mRNA) transcripts, which in turn are used as the templates to create polypeptide proteins during translation.

During the first step of mRNA synthesis, a complex of initator proteins assembles on the promoter, including RNA polymerase and a collection of transcription factors (Chang and Jaehning 1997; Hampsey 1998; Krishnamurthy and Hampsey 2009; Vannini and Cramer2012). The TATA-binding protein (TBP) is critical for initiating transcription (Cox et al. 2010; Hampsey 1998). When present, an upstream transcription factor IIB (TFIIB) binding recognition element (BRE) or a downstream promoter element (DPE) is utilized as part of the core promoters. Several other regulatory elements and transcription elements are responsible for controlling polymerase accessibility to a particular gene and may impact transcription initiation. A mediator complex protein must also be recruited to drive the synthesis of mRNA, through interactions with transcriptional activators bound to enhancer sequences. When all necessary proteins are recruited, the entire assembly is termed a "holoenzyme," and transcription is ready to begin.

Once initiated, RNA polymerase II begins generating the chain of the RNA sequence, complementary to the template DNA strand (Krishnamurthy and Hampsey 2009). New ribonucleotides used to extend the growing transcript are held together by phosphodiester bonds. Transcription continues until termination is signaled by mRNA sequence elements, leaving a completed mRNA construct, which will then be transported out of the nucleus to be translated into a functional protein.

The molecular mechanism of translation has also been well established (Andersen et al. 2004; Cox et al. 2010; Pestova 2001). The process of translation begins with the

assembly of the ribosomal subunits. With help from eukaryotic initation factor 1 (eIF1), the small ribosomal subunit locates and binds the 5'-AUG start codon (AUG) on the mRNA (Figure 3). Once recruited, eukaryotic initiation factors 2 (eIF2) joins the small ribosomal subunit. eIF2 binds to the methionine-tRNA. This initiation step is highly regulated by eIF1, which governs whether or not translation will occur, based on whether or not it is able to bind the start codon. Once eIF2 is bound, the large 80S ribosomal subunit is recruited and eukaryotic initiation factor 5B (eIF5B) facilitates the joining to the small subunit with the help of eukaryotic initiation factor GTPase (eIF6), thereby completing the formation of the initiation complex. This newly formed ribosomal complex contains three tRNA binding sites, A, P, and E. The start codon lands in the P site at translation initiation. In the next step of translation, coded tRNAs will bind to the anticodon sequences in the A site as new amino acids become integrated into the growing polypeptide chain.

Elongation factors are responsible for the extension of the polypeptide protein chain (Figure 3) (Andersen et al. 2004; Cox et al. 2010; Dever and Green 2012). Eukaryotic elongation factor 1α (eEF1α) transports an aminoacyl-tRNA to the ribosome. If the charged, amino-acylated tRNA has the complementary anticodon sequence to the exposed mRNA codon, then the charged tRNA may bind to the A site and forms a peptide bond to the tRNA bound to the P site of the ribosome. In the next step, eukaryotic elongation factor 2 (eEF2) translocates the large ribosomal subunit, freeing the A site, and moving the existing tRNAs from the P and A sites to the E and P sites, respectively. At this point, a rapid ejection of the deaminated-tRNA from the E site takes

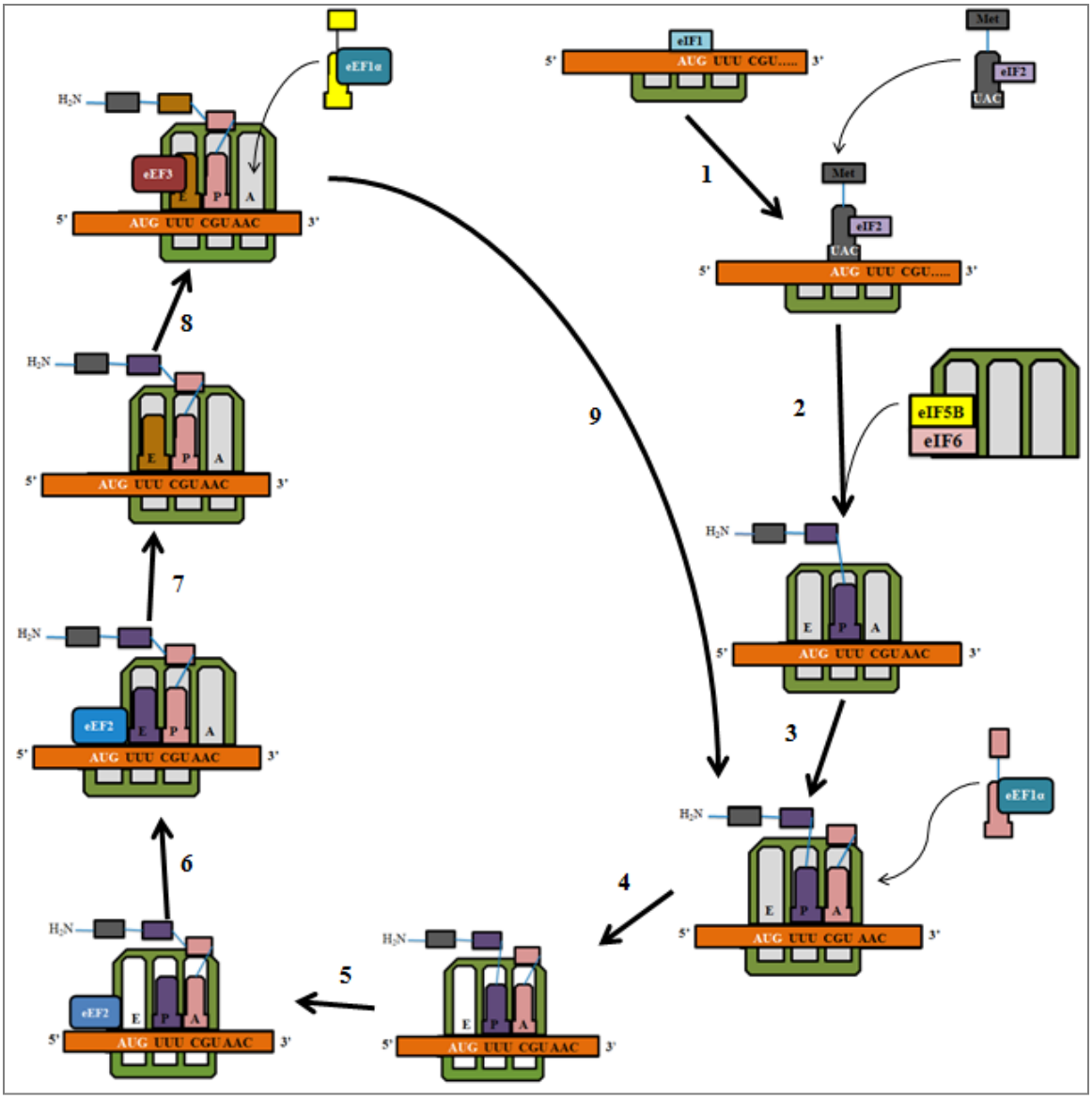

Figure 3 – **The steps of eukaryotic translation**. (1) eIF1 binds the start codon and the small ribosomal subunit is recruited to the mRNA transcript. eIF2 recruits the first amino acid to the P site of the ribosome. (2) eIF5B and eIF6 recruit the large ribosomal subunit to complete the ribosomal complex necessary for translation. Once the large ribosomal subunit binds, eIF5B and eIF6 dissociate. (3) eEF1α recruits aminoacyl tRNA to the A site. (4) eEF2 binds the complex. (5) A peptide bond is formed between the amino acids bound to the tRNA in the P site and the A site. (6) eEF2 binds and translocates the large ribosomal subunit, shifting the tRNAs from the A and P sites to the P and E sites, respectively. (7) The fully translocated ribosome. (8) eEF3 binds the uncharged tRNA and (9) removes the uncharged tRNA from the E site, while eEF1α recruits a new aminoacyl tRNA to the A site. This peptide elongation process continues from step 3 until translational termination is reached.

place, while at the same time, eEF1α transports a new, charged tRNA to the A site. The peptide bond formation, ribosomal translocation, and cycling of the tRNAs continues until a stop codon is reached, at which point translation is terminated via a release factor.

The development of anti-fungal drugs has been challenged by the fact that in the case of human mycoses, both the pathogen and the host are eukaryotic organisms, so both have similar cellular structures and utilize the same proteins for gene expression. To develop a highly efficacious anti-fungal drug, a unique structure must be identified in fungi. Further, since fungal pathogens are present in all four phyla, an ideal, broad-spectrum anti-fungal drug would target a structure unique to all fungi yet absent from humans. In particular, if a protein essential for gene expression in the pathogen could be targeted, these cells would not be able to reproduce with viability, thus terminating further growth and proliferation of the infecting agent while leaving the host unaffected by the drug. Using such a model, patients could conceivably be cured of fungal infections once all pre-existing cells colonizing the patient die upon drug-therapy-induced termination of gene expression mechanisms critical for fungal cell survival. Identifying such a structure has been laborious effort of many researchers over the past few decades (Deepe 1997; Marr 2004).

In the cells of lower eukaryotic organisms, including fungi, there is a unique protein, eEF3, which is essential for cell viability (Andersen et al. 2004; Dasmahapatra and Chakraburtty 1981; Qin et al. 1990). Deletion of this protein prevents cellular viability among organisms that contain it. Based on experimental evidence, eEF3 is

hypothesized to be involved with translation, in which the uncharged tRNA is removed from the ribosomal E site, allowing the ribosome to shift (Anand et al. 2003; Andersen et al. 2006). This, in turn, aids eEF1α in its delivery of the next charged aminoacyl tRNA in the sequence to the elongating ribosome, allowing translocation to occur once again, thereby continuing the elongation process for the growing polypeptide chain. eEF3 has two ATP binding cassettes (ABC cassettes) that allow it to perform its function through ATP hydrolysis, which yields the energy required for ejecting the uncharged tRNA from the E site of the ribosome (Anand et al. 2006; Sarthy et al. 1998; Sasikumar and Kinzy 2014). In fact, in the search for a conserved region of eEF3 that may be therapeutically targeted, Sasikumar and Kinzy found a chromodomain-like insertion within one of these ABC cassettes that is vital to the protein's function (2014).

Additional research has discovered that eEF3 may also couple with Cch1, a Ca^{2+} channel of the plasma membrane, implicating that it serves functions outside of translation (Liu and Gelli 2008). Its exact role here has not yet been identified, but it is hypothesized to be involved in localization of Cch1 to the plasma membrane.

1.4 Regulations Controlling Gene Expression

The processes of transcription and translation are highly regulated in order to sustain viable cellular growth and prevent an over-expenditure of energy stores on unnecessary RNA or protein products (Cox et al. 2010; Chang and Jaehning 1997). In transcription, regulatory DNA sequences bind transcription factors to either permit or prohibit gene expression. TBPs also regulate transcription initiation by binding the DNA sequences to positioning transcriptional proteins as they assemble on the promoter region.

All necessary transcription factors that constitute the holoenzyme must be present to aid Pol II in accurately transcribing genes. These include TBP, TFIIB, TFIID, TFIIF, TFIIE, TFIIH, general transcription factors, and co-activators. Mediator proteins must be present to drive initiation. Transcription factors regulate this step by positioning necessary proteins on the DNA chain.

In addition to the need for transcriptional proteins, the synthesis of RNA is also controlled by the accessibility of such proteins to the template DNA strand. This is impacted by epigenetic gene activation or silencing, or by DNA chromatin coiling (Jenuwein and Allis 2001; Roy et al. 2014). Chromatin structure is regulated by histone modifications including acetylation, methylation, phosphorylation, ubiquitination, and deacetylation. In addition, RNA polymerase may be blocked from accessing the template DNA strand due to the presence of certain compounds that form chemical interactions with transcriptional proteins, known as corepressors, coactivators, or chromatin modiifiers (Kumar et al. 2005; Vetter 1997). Such chemicals are typically small in size, and often contain ring structures to maximize intermolecular interactions with the amino acids of the transcriptional machinery. Examples include steroidal hormones or small cyclic polypeptides, such as α-amantin found in the mushroom *Amanita phalloides* (Cox et al. 2010; Vetter 1997).

Translation may be regulated at its initiation steps (Sonenberg and Hinnebusch 2009; Cox et al. 2010). Such control is particularly important for the regulation of the expression of large proteins that must be rapidly assembled, immediately as signals are received by a particular cell. Initiation may be regulated by RNA folding that blocks the

ribosomal scanning mechanisms used to find and bind the small ribosomal subunit. The same regulation mechanism may be induced by binding of microRNA segments to the 3' end of the untranslated region near the start codon.

1.5 Eukaryotic Elongation Factor 3 – the Next Anti-Fungal Drug Target?

It is particularly important to note that eEF3 is absent from the mammalian genome. The role of eEF3 is hypothesized to be fulfilled by other translational proteins in higher eukaryotes that lack the eEF3 protein. This makes it a strong candidate for a potential drug target for treating mycoses in humans (Kubitschek-Barreira et al. 2013). Since there is no eEF3 homolog in humans, the risk of the anti-eEF3 drug harming human cells is minimized. Systemic drug toxicity will likely be very low, given the low abundance of commensal fungi cells in humans, relative to human cells and the plentiful bacterial colonies in the digestive tract.

Another benefit of using eEF3 as a drug target is that it is essential for viability in a broad range of organisms. The protein is present and essential in all fungi; if an anti-eEF3 drug were developed, it would likely be beneficial in treating mycoses caused by any fungi. Of course, this hypothesis is not guaranteed, and it would need to be tested in further experiments, as *in vivo* effectiveness may vary based on slight alterations in the eEF3 protein structure from species to species. Ideally, a highly conserved region of the eEF3 protein may be targeted that is present among all pathogenic fungi, in order to allow for the development of a broad-spectrum, minimally-toxic anti-fungal agent.

Before eEF3 may be used as a drug therapy target, a better understanding of the

protein's gene sequence, its physical structure, and its functional role needs to be ascertained. Previous studies have determined the genomic sequence, protein size, and protein structure of eEF3, and ongoing studies are currently working to elucidate critical residues within the protein (Anand et al. 2006; Andersen et al. 2004; Dasmahapatra and Chakraburtty 1981; Qin et al. 1990; Sarthy et al. 1998; Sasikumar and Kinzy 2014). Another area for further study is to determine the functional conservation and divergence of the protein, which may help further elucidate how the protein is able to be absent from mammalian species. Additionally, studies of the functional conservation of eEF3 will help determine whether antifungal agents that target this protein would be narrow or broad spectrum compounds, based on whether compounds targeting eEF3 bind only to eEF3 of a particular subset of fungal pathogens or to all fungal pathogens. The ability of drug compounds to bind eEF3 is governed by structural differences, which can be inferred by functional analysis.

Eukaryotic translation factors eEF1 and eEF2 are both highly conserved (Dasmahapatra and Chakraburtty 1981). This is a testament to the evolutionary selection for universal DNA encoding and expression. Such universality has long been favored by the horizontal gene transfer mechanisms which organisms have used to share their genes and evolve since the earliest ancestral cells (Koonin 2014). It stands to reason that the structure and function of this third translation protein, eEF3, would also be highly conserved among those organisms that rely upon it for viability.

Previous research has analyzed the conservation of eEF3. In one experiment, functional conservation was tested between yeast species of two different phyla

(Colthurst et al. 1992; Blakely et al. 2001). The Tuite lab found that eEF3 from *Cryptococcus neoformans* (*C. neoformans*), a dimorphic species within the fungal phylum *Basidiomycota*, was homologous to eEF3 from *S. cerevisiae*, a species within the phylum *Ascomycota*. Blakely's and his colleagues' findings expanded these results to show that eEF3 from *C. neoformans* supported only limited viability when transformed into *S. cerevisiae* (budding yeast). The budding yeast containing the transformed product had a slower growth rate and smaller colonies than that of untransformed budding yeast. Therefore, there was poor conservation of eEF3 between the two phyla, which could be caused by possible structural or functional differences present among eEF3 from different lower eukaryotic species.

Previous research in Dr. Dunaway's laboratory investigated the conservation of eEF3 between two yeast species, both of phylum *Ascomycota*. The two species being investigated were *Schizosaccharomyces pombe* (commonly known as fission yeast) and *S. cerevisiae*. The budding yeast strain used contained an exogenous copy of its *S. cerevisiae* eEF3 (ScEF3). When eEF3 from *S. pombe* (SpEF3) was transformed into budding yeast, SpEF3 was able to support cell viability in a budding yeast strain that had expelled its exogenous ScEF3 gene (Colmer 2013). These data showed that between these two yeasts of the same phylum, the eEF3 gene is functionally conserved. This experiment was also very useful in creating a framework for a research method for testing the functional conservation of a protein. This technique could easily be replicated using an eEF3 gene insert from virtually any lower eukaryote and transforming it into *S. cerevisiae*, a model lower eukaryotic organism that can be easily manipulated in the lab

for genetic studies.

1.6 The Functional Conservation of eEF3 Beyond Yeast

This project sought to widen the scope of knowledge regarding eEF3 by studying the functional conservation of the protein between unicellular green algae (*C. rheinhardtii*), which is a unicellular, non-fungal, lower eukaryote, and budding yeast (*S. cerevisiae*). These two species are from more divergent phyla than the species investigated in any previously published study of the functional conservation of eEF3 (Table 1) (UniProt 2014; NCBI - BLAST). Notably, each of these species have an initial DNA segment with alignment scores less than 40 when compared to eEF3 of *S. cerevisiae*, which indicates that the beginning of the gene is likely not conserved. For *C. reinhardtii*, this region is 64 amino acids long; for *S. pombe*, this is only 4 amino acids long; for *C. neoformans*, this is 20 amino acids long.

This study sought to answer whether eEF3 from green algae is able to support viability in budding yeast. If it is, how is the growth rate of transformant cells affected? Further research on eEF3 may further elucidate why the gene is lost in the human genome. Is there another protein that performs the same function in human cells, allowing eEF3 to be evolutionarily lost?

This project used the model system that was previously utilized by Sarah Colmer in Dr. Dunaway's lab to analyze the complementation of fission yeast eEF3 in budding yeast cell cultures (2013). For this experiment, the SpEF3 used by Sarah Colmer was replaced by that of the eEF3 gene from *C. reinhardtii* (CrEF3), to make a low expression

Table 1 – Amino acid alignment data for eEF3 compared to *S. cerevisiae*

Species	Amino Acid Length	% Identity	Similar Positions	% Homology
S. cerevisiae	1044	---	---	---
S. pombe	1047	61.101%	254	85.361%
C. neoformans	1055	44.372%	300	72.808%
C. reinhardtii	1053	42.486%	299	70.881%

plasmid containing CrEF3 (LE-CrEF3). The plasmid shuffle scheme shown in Figure 4 outlines the method used. The isolated CrEF3 gene was inserted onto a plasmid with selection markers specific to selective growth media. These markers promote expression of LE-CrEF3 within *S. cerevisiae* cells. The *S. cerevisiae* cells had their copy of eEF3 on an extra-chromosomal plasmid with different selection markers that enables growth media selection for transformed cells to discard their own, exogenous ScEF3 plasmid (Ex-ScEF3). If CrEF3 is able to support viable growth of *S. cerevisiae* cells, the transformed yeast cells would be able to grow on the final selection plate using media toxic to cells relying upon Ex-ScEF3. Western blot analysis was conducted to measure changes in eEF3 protein expression levels. By making use of an incorporated amino acid tag present on the inserted CrEF3 gene, western blot analysis elucidated how much of the eEF3 expression was coming from *C. reinhardtii*'s LE-CrEF3 and how much came from *S. cerevisiae*'s Ex-ScEF3.

Since eEF3 is unique to a relatively small group of organisms, it was hypothesized that it would have high functional conservation and would successfully complement between *C. reinhardtii* and *S. cerevisiae*, despite the larger taxonomic divergence gap of the species. Based on observed trends seen in past research (Blakely et al. 2001; Colmer 2013), it was likely that the transformed gene would support viable *S. cerevisiae* cell growth but at an even slower cell growth rate. The difference in cell growth rate and cell size between control *S. cerevisiae* strains and transformant *S. cerevisiae* strains containing CrEF3 would perhaps be even larger than that measured during Blakely's and his colleagues' research, since the eEF3 to be analyzed came from

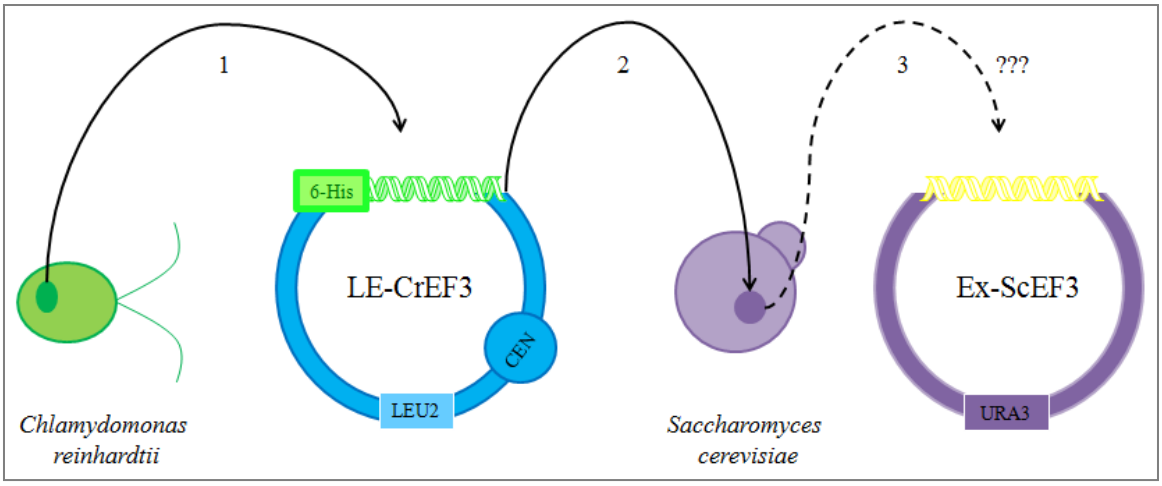

Figure 4 – **Plasmid shuffle scheme for transforming CrEF3 into *S. cerevisiae***. In step 1, the CrEF3 gene was isolated from *C. reinhardtii* and inserted onto a low expression plasmid to make LE-CrEF3. In step 2, LE-CrEF3 was transformed into *S. cerevisiae* cells. In step 3, tests were used to determine if *S. cerevisiae* cells were able to rely solely on the expression of CrEF3 to maintain cellular viability and lose their exogenous copy of ScEF3 (Ex-ScEF3).

an even more taxonomically divergent species (2001). The results of this research provide additional understanding of the degree of functional conservation of the eEF3 protein by testing expression of eEF3 transformed between two species that are more taxonomically divergent than have been tested in the past. In working towards identifying eEF3 as a potential anti-fungal drug target, this research answers whether or not eEF3 is a highly conserved protein that can be targeted in any lower eukaryote using one approach. Conversely, this research also determines whether this protein unique to lower eukaryotes has too much variation within the small group of species that contain it, which may limit the applicability of future anti-fungal drugs targeting the protein.

2. MATERIALS AND METHODS

2.1 Low-Expression Plasmid Investigation

Extraction and Amplification of CrEF3

Collaborators from Dr. Terry Kinzy's laboratory at Rutgers University provided pure, amplified CrEF3 gene fragment. They extracted the gene from a phage library containing the full CrEF3 gene, pre-marked with a six-histidine tag, within a portion of the *C. reinhardtii* genome. They amplified the gene of interest using a polymerase chain reaction (PCR). PCR is a commonly used molecular biological technique to rapidly copy specific regions DNA. It requires a mix of template DNA (which will be specifically amplified), primers complementary to the template DNA in both the forward and reverse directions for double-stranded DNA, a thermostable DNA polymerase enzyme to extend the primer complementary to the template strand, and nucleotides to be added by the polymerase to the growing DNA strand. In PCR reactions, the primers selected determine which region of DNA will be copied.

For this experiment, the PCR conducted required forward and reverse primers (Table 2) specific to CrEF3 in order to remove excess terminal DNA that was present in the phage library. A Phusion ® High-Fidelity PCR kit (which includes nucleotides and polymerase enzymes) and magnesium chloride were also used (New England BioLabs 2015e). This method of PCR was used since the Phusion PCR kit is very accurate, and magnesium chloride further optimizes the performance of the Phusion DNA polymerase included in the kit (Sigma-Aldrich 2014). The PCR reaction was run according to the

Table 2 – PCR Primers for CrEF3 Amplification

Name	Number of Bases	Sequence	Notes
CrEF3 Reverse	33	5'- CTT TTA CTC GAG TTA GCA CTC CTC CTC GGA GTC -3'	
CrEF3 Forward	54	5'- ATT GTT GGA TCC ATG CAT CAT CAT CAT CAT CAT TCT GCC GAC GCC GAC GTT AAC -3'	Underlined region is complementary to 6-His Tag on the CrEF3 gene.

Table 3 – PCR Cycle Specifications

	Initiation	Cycle, 35 times			Hold
Temperature (°C)	98.0	98.0	59.0	72.0	72.0
Time (min:sec)	1:00	0:30	0:30	2:00	7:00

cycle parameters specified in Table 3. The resulting PCR product was transported to Dr. Dunaway's lab and stored in a 10°C refrigerator to be used for the rest of the experiment. An agarose gel was run to confirm the appropriately sized gene was present. A 12 kilobase (1 kb is 1,000 nucleotides in length) DNA ladder from New England BioLabs, Inc., was used to gauge the band size of the digested PCR product.

Digestion and Ligation of CrEF3 into a Low-Expression Plasmid

For the purposes of molecularly cloning the eEF3 gene of interest into *S. cerevisiae* cells, the eEF3 gene needed to be inserted onto a plasmid to allow budding yeast cells to uptake the foreign DNA. A LEU2-marked CEN plasmid called pTK328, shown in Figure 5, was chosen for this purpose. This plasmid has a reasonable base pair length, allowing the eEF3 gene of interest to be inserted while maintaining a sufficiently small size to allow the plasmid to be taken up by the *S. cerevisiae* cells. Additionally, the plasmid has the appropriate selection markers to allow for cloning manipulation on selective media plates. The plasmid has an ampicillin resistance gene and origin of replication, allowing for initial isolation of ligated plasmids using DH5α *E. coli* cells. The plasmid also has a leucine marker (LEU2), which is unique from the uracil marker (URA3) present on the exogenous plasmid copy of ScEF3 gene, thereby allowing growth media selection to favor the expression of the foreign CrEF3 gene by the *S. cerevisiae* cells.

The sequences for the linear *C. reinhardtii* gene product and the pTK328 plasmid to be used were compared, and they were found to both contain binding sites for the *Bam*HI and *Xho*I enzymes. These enzymes were desirable because they create

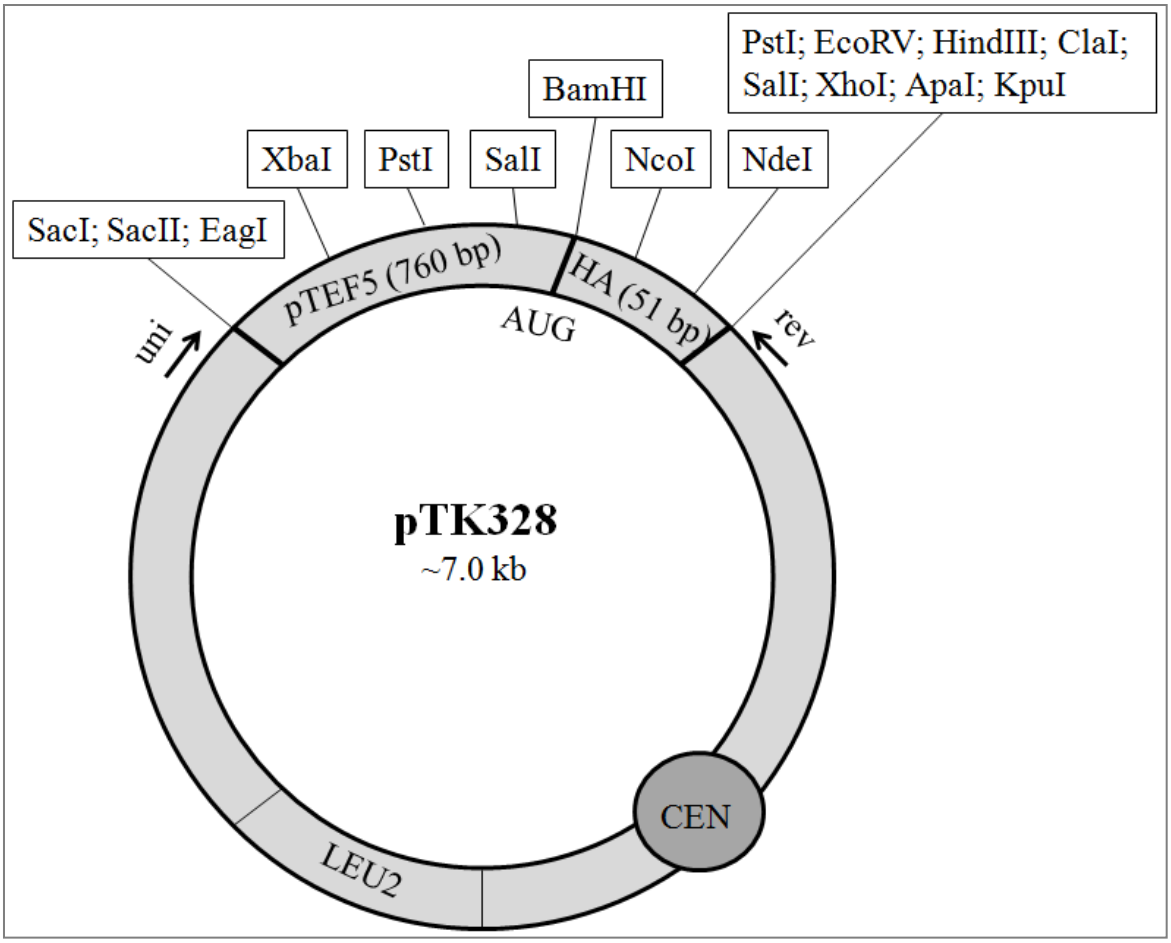

Figure 5 – The pTK328 plasmid. This 7.0 kb plasmid has restriction binding sites for *Bam*HI and *Xho*I. A LEU2 marker allows selection during transformation steps, and a CEN promoter maintains low expression levels. The plasmid also has an origin of replication and ampicillin-resistance gene for bacterial selection.

complementary sticky ends for ligating two genes together, as shown in Table 4 (New England Biolabs 2015b,d,f). Upon receiving the amplified CrEF3 gene product from Dr. Kinzy's lab, both the CrEF3 gene product and a selected LEU2-marked CEN plasmid were individually digested by mixing the reagents indicated in Table 5 and incubating at 37°C for 90-120 minutes. Double digestion using these two enzymes creates sticky, complementary ends of DNA on both the CrEF3 gene insert and the plasmid. This step ensures that the eEF3 gene inserts in a uniform direction onto the plasmid, versus the alternative blunt cut or single enzyme digest, either of which would allow the gene to insert in both the forward and reverse manner.

The pTK328 plasmid also underwent digestion using calf intestinal phosphatase (CIP) to remove the 5' phosphate (Table 5). CIP was added to the double digestion reaction mixture and incubated at 37°C for one hour. This inhibits the plasmid from ligating with itself, which would create an "empty plasmid" lacking the CrEF3 gene insert, by dephosphorylating the protruding 5' end of DNA (New England BioLabs 2015a). CIP also improves the likelihood that the eEF3 gene may be ligated into the plasmid. An agarose gel was run to verify that the eEF3 gene of interest was successfully amplified and digested, according to the base-pair size of the eEF3 gene, which is around 3.2 kb.

The double-digested plasmid DNA was extracted from the agarose gel and purified using a QIAGEN QIAQuick Extraction kit and following the included directions. The double-digested CrEF3 gene also underwent a QIAQuick Purification using a QIAGEN kit, following the double digest. A NanoDrop analysis was run on both the

Table 4 – Restriction enzyme sequences

Restriction Enzyme	Cleaved Sequence
*Bam*HI	5'…G*G A T C C…3' 3'…C C T A G*G…5'
*Sac*I	5'…G A G C T *C…3' 3'…C*T C G A G…5'
*Xho*I	5'…C*T C G A G…3' 3'…G A G C T *C…5'

Table 5 – *Bam*HI and *Xho*I double digestion specifications

Component	Volume (µL)		
	Digest for pTK328 plasmid	**Digest for CrEF3 gene insert**	**Diagnostic Digest After DH5α Transformation**
Water	8.0	1.5	8.0
Buffer 4	4.0	2.5	4.0
BSA	1.0	1.0	1.0
DNA (as indicated)	8.0*	19.0*	5.0
***Bam*HI**	1.0	0.5	1.0
***Xho*I**	1.0	0.5	1.0
CIP	1.0	---	---

*Volumes differed based on relative nucleic acid concentrations observed by NanoDrop™ nucleic acid concentration analysis.

plasmid and the eEF3 double digest extracts to calculate nucleic acid concentrations and to determine the volumes for each that was necessary for a ligation at a 1.8:1 ratio of CrEF3 to plasmid DNA. The purified CrEF3 was inserted into the plasmid using a Quick Ligation Protocol from New England Biolabs to make LE-CrEF3. The following alterations to the protocol were made based on what had been successful for Sarah Colmer's research (New England Biolabs 2015c; Colmer 2013). Components were mixed according to the protocol, using a 10x ligase buffer and T4 DNA Ligase. Once everything had been added to the reaction tube, the mixture was incubated for 20 minutes at room temperature, rather than the 5 minutes indicated in the protocol, before chilling and storing at -20°C.

Transformation into DH5α Competent Cells

The newly ligated LE-CrEF3 plasmid was transformed into DH5α competent *Escherichia coli* (*E. coli*) cells using Subcloning Efficiency DH5α Competent Cells and Protocol from Invitrogen. Two positive controls were used to ensure all reagents were viable – one using pUC19 and one using uncut CEN plasmid. The volumes of DNA used are shown in Table 6. Transformant cells were mixed with 250.0 µL Luria Broth-ampicillin (LB-amp) liquid media and plated on 50 µg/mL LB-amp plates, pre-warmed to 37°C. Transformant plates were incubated overnight at 37°C. Resultant colonies were inoculated into 5 mL aliquots of LB-amp liquid media (of the same concentration as the plates) and incubated overnight at 37°C.

Plasmid DNA was purified from a sample of each inoculated LB-amp culture using a QIAPrep Spin Miniprep Kit (QIAGEN). Double digests were run using *Bam*HI

Table 6 – DNA volumes added for DH5α Transformation

Reaction #	DNA	Volume DNA added (µL)	Volume Plated (µL)
1	Ligation product	5.0	50.0
			200.0
2	Ligation product	15.0	50.0
			200.0
3	pUC19	2.5	10.0 (diluted in an additional 90.0 µL liquid LB-amp media)
			100.0 (undiluted)
4	Uncut pTK328 vector DNA	2.0	10.0 (diluted in an additional 90.0 µL liquid LB-amp media)
			100.0 (undiluted)

and *Xho*I and analyzed using gel electrophoresis to determine if ligation was successful, based on whether CrEF3 was present in the DH5α cells (Table 5). From successful DH5α cell transformants that contained CrEF3, extracted plasmid DNA was stored in preparation for transformation into *S. cerevisiae*. Additionally, 30% glycerol stock cultures (named KBB1-1 and KBB1-2) were prepared for successful DH5α transformants that contained the ligated LE-CrEF3 plasmid stored at -80°C.

***Transformation into* S. cerevisiae**

An *S. cerevisiae* strain that had previously been modified to have its eEF3 gene on an exogenous URA3-marked plasmid (Ex-ScEF3) was inoculated onto synthetic dropout plates lacking uracil (SD-Ura plates) and incubated at 30°C. One colony was inoculated into a Erlenmeyer flask containing 50 mL of liquid SD-Ura media. The culture was grown in a 30°C incubator while shaking at 170 rotations per minute (rpm) until the optical density of a 1 mL aliquot at 600 nm reached 0.700, as determined by a BioMate 3 v2.100 spectrophotometer. This optical density at this wavelength corresponds to the exponential growth phase of *S. cerevisiae* cells (data not shown). The remaining liquid culture was centrifuged at 2800 rpm for 15 minutes to pellet the cells present, and the SD-Ura media was removed and discarded. Cells were then rinsed in distilled, de-ionized water. Cells were pelleted again and the aqueous supernatant was discarded. This rinse was repeated twice. The cells were resuspended in 1.0 mL of 100 mM lithium acetate, pelleted at 13,200 rpm for 15 sec, and the supernatant was discarded. The cell pellet was resuspended in 400 μL of 100 mM lithium acetate and divided into four aliquots of 50 μL each. The remaining 200 μL of prepared cells were discarded, as

they were not needed. The cells were pelleted, and the lithium acetate supernatant was removed.

Cells were transformed by mixing the reagents shown in Table 7, in the order listed. Lithium acetate and polyethylene glycol were used to make the *S. cerevisiae* cell membrane more permeable, in order to allow easy uptake of LE-CrEF3. Two experimental tests were run, each using miniprepped, pure DNA from the DH5α transformant cultures KBB1-1 and KBB1-2. A positive control was performed using undigested LEU2-marked CEN plasmid. A negative control was run using no DNA. Based on the transformation technique refined by Gietz and Woods in 2002, polyethylene glycol and lithium acetate are both known to improve transformation efficiency (Kawai et al. 2010). Single-stranded DNA serves as a carrier for bringing foreign DNA into the *S. cerevisiae* cells. Following a one-minute vortex and a thirty-minute incubation at 30°C on a roller, 40 μL dimethyl sulfoxide (DMSO) was added, and the cells were heat shocked at 42°C for fifteen minutes. DMSO is known to improve the success of transformation into *S. cerevisiae* cells (Hill 1991). Cells were pelleted and the supernatant from the transformation reaction was discarded. Transformant cells were resuspended in 200.0 μL SD(-Ura/-Leu) liquid media and plated on SD(-Ura/-Leu) media. Transformant plates were incubated for up to three days at 30°C.

Growth Media Selection of* S. cerevisiae *cells containing CrEF3

Sixteen transformant colonies from the KBB1-1 transformation, sixteen transformants from the KBB1-2 transformation, and eight transformants from the positive control pTK328 transformation were re-streaked onto SD-Leu plates to encourage the

Table 7 – *S. cerevisiae* transformation reaction mixtures

Transformation #	Component	Volume (µL)
Added to all 4 transformation mixtures	50% weight per volume (w/v) poly-ethylene glycol	240.0
	Lithium acetate (1.0 M)	36.0
	Boiled herring sperm single-stranded DNA (ssDNA) at 2 mg/mL	5.0
1 only	Miniprepped DNA of KBB1-1	15.0
2 only	Miniprepped DNA of KBB1-2	15.0
3 only	Miniprepped DNA of undigested pTK328 (positive control)	15
4 only	No DNA (negative control)	---

yeast to maintain and use CrEF3 genes preferentially over their exogenous ScEF3 gene. Selection plates grew for up to three days at 30°C. These selected forty cultures were copied onto a second SD-Leu plate. One colony from each of the forty cultures on the second SD-Leu plates were copied onto SD-Ura plates to qualitatively determine whether expression of the exogenous ScEF3 plasmid (which contains the URA3 marker) was still taking place. Additionally, one colony from each of the forty cultures was re-streaked onto a third SD-Leu plate.

As the absolute test to determine whether CrEF3 can support viable *S. cerevisiae* cells, select colonies from the third set of SD-Leu plates were streaked onto plates containing the drug compound 5-fluoroorotic acid (5-FOA). These plates were provided by collaborators at the Kinzy lab. On 5-FOA plates, a URA3 marker prohibits cell growth in yeast expressing a URA3 gene (Life Technologies 2015). *S. cerevisiae* cells are only be able to survive if they take up, express, and use the LE-CrEF3 preferentially, kicking out its own exogenous copy of ScEF3, which is on a URA3-marked plasmid. A positive control of *S. cerevisiae* expressing SpEF3 was tested on the 5-FOA plates as well (Colmer 2013). A negative control using untransformed *S. cerevisiae* was also tested on the 5-FOA plates. Thirteen experimental colonies were tested which contained the CrEF3 gene: six colonies came from thrice re-streaked KBB1-1 cultures and seven colonies came from thrice re-streaked KBB1-2 cultures.

Protein Extraction and Western Blot Analysis

Cells were pelleted from the same cultures that had been tested on 5-FOA plates and lysed to analyze the protein expression levels present. Liquid media cultures were

grown to optical density of around 0.700, as read by a BioMate 3 v2.100 spectrophotometer at 600 nm, for untransformed *S. cerevisiae, S. cerevisiae* containing the empty low expression plasmid without CrEF3, and from *S. cerevisiae* transformants containing LE-CrEF3 from both KBB1-1 and KBB1-2. Once the optical density was reached, 15 mL cultures were pelleted at 2800 rpm, growth media was removed, and cells were re-suspended in 5.0 mL distilled, deionoized water (ddH_2O) and frozen overnight.

The next day, cells were pelleted, the ddH_2O was removed. Cells were resuspended in 1.0 mL phosphate buffered saline (PBS) and transferred to an atom smasher tube. Cells were pelleted at max speed at 5°C, and excess PBS was removed. Cells were resuspended in lysis buffer (250 μL from a 10 mL PBS solution containing one Roche Complete Mini EDTA-free protease inhibitor cocktail tablet and 100 μL PMSF). Autoclaved glass beads were added to the meniscus. This mixture was cycled in an atom smasher at 6.5 speed for 20 seconds, followed by two minutes on ice. This cycle was repeated two times. Atom smasher tubes were pierced, placed in a eppendorf tube, and centrifuged at 2000 rpm for 90 s at 5°C to isolate cellular components from glass beads. The atom smasher tube containing glass beads was discarded, and the eppendorf tube was spun for 10 minutes at 13,200 rpm to separate the proteins in solution from the additional cellular components. The resultant supernatant (~200 μL total), containing isolated cellular proteins, was transferred to a fresh eppendorf tube.

A Bradford Assay was run to determine protein concentration (Bio-Rad 2013). Bovine serum albumin (BSA) standards were used to calibrate the spectrophotometer at 595 nm with known protein concentrations ranging from 0.0 mg/mL to 0.8 mg/mL.

Isolated protein samples were diluted using 2X GSB and again diluted 1:20 to yield a 1.0 mL aliquot to read the protein concentration using the BioMate 3 v2.100 spectrophotometer at 595 nm.

Additionally, two western blots were run to test levels of protein expression using protein extracted from seven unique colonies, following a protocol from Bio-Rad. The first western blot used a primary antibody against the 6-histidine tag to measure the expression of eEF3 coming explicitly from the six-histidine-tagged CrEF3. The second western blot used a primary antibody against eEF3 to measure the total expression level of eEF3 from both CrEF3 and ScEF3. This antibody is known to have no interaction with the other elongation factors (Dasmahapatra and Chakraburtty 1981).

2.2 High-Expression Plasmid Investigation

Due to the increased divergence of the two species and limited CrEF3 protein expression seen in transformant *S. cerevisiae* cells containing the LE-CrEF3 plasmid, the experiment was repeated using a high expression, 2μ plasmid called pTK372 in order to obtain higher expression levels (Figure 6). This required one major change to the above design: when performing the double digest to ligate CrEF3 into the plasmid, the endonuclease enzyme *Sac*I (obtained from New England BioLabs) was used in place of *Bam*HI, as the 2μ plasmid lacks sufficient complementarity to the *Bam*HI sequence. This new plasmid construct was ligated using the same method as above to create the high expression plasmid containing CrEF3 (HE-CrEF3).

CrEF3 was isolated from KBB1-1 and KBB1-2 *E. coli* strains, and pTK372 was

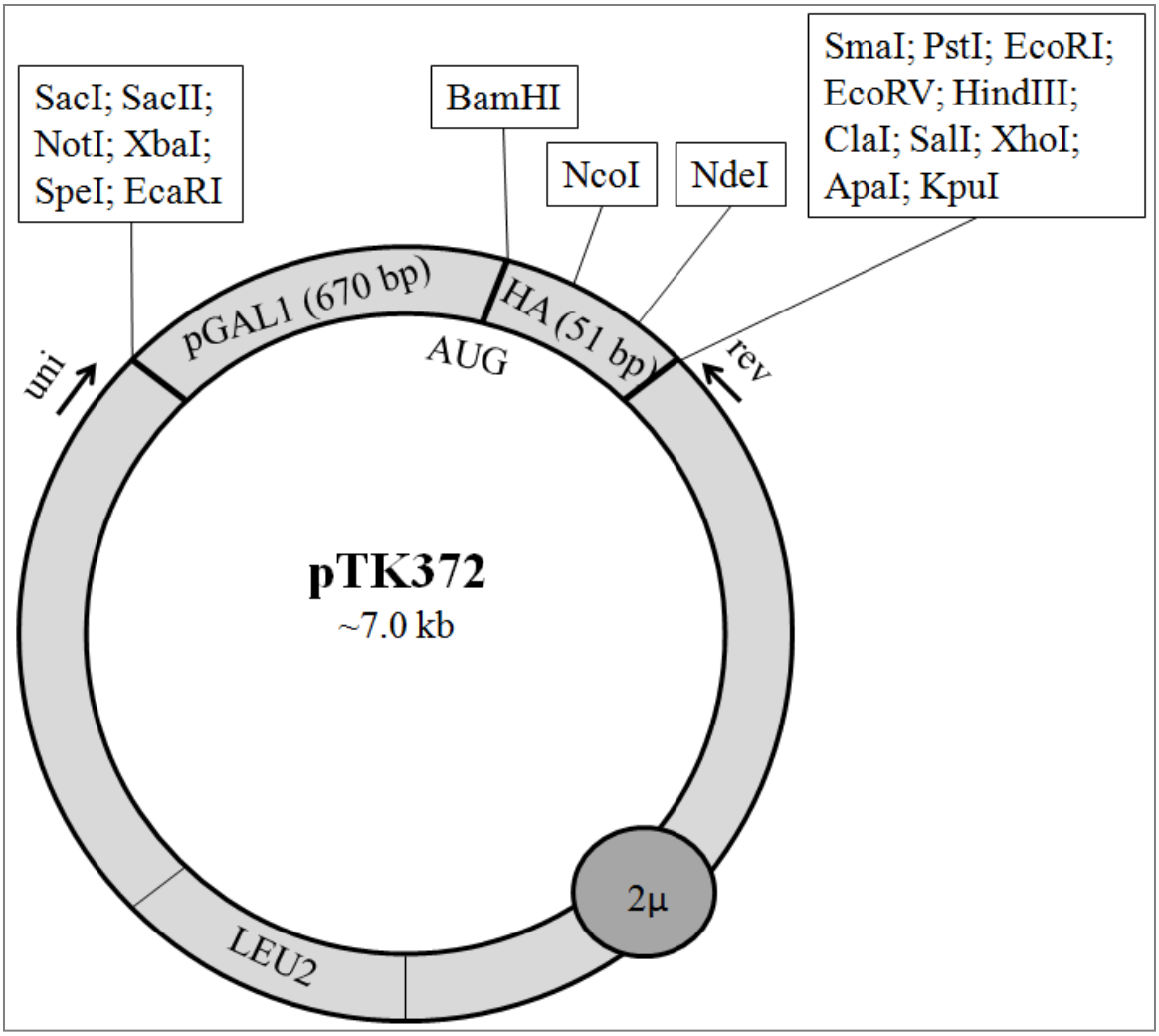

Figure 6 – The pTK372 plasmid. This 7.0 kb plasmid has restriction binding sites for *Sac*I and *Xho*I. There is also a LEU2 marker for selection and a 2µ promoter to maintain high expression levels. The LEU2 marker allows for selection during transformation steps and growth media manipulation. The 2µ marker encodes for a higher copy number.

isolated from a separate *E. coli* strain. Cells were miniprepped to yield pure plasmid DNA. A glycerol stock was also prepared for pTK372 to store in the -80°C freezer (labeled TKB372). Both isolated plasmids were double digested to verify that the *Sac*I and *Xho*HI restriction endonucleases would successfully cleave the DNA in the desired locations. Notably, as a result of the change in restriction enzymes, it was expected that the CrEF3 gene would retain the 760 bp pTEF5 gene from the low expression plasmid.

Steven Silva, another undergraduate researcher of Dr. Dunaway's lab, performed the gel extraction of the double-digested CrEF3 gene and pTK372 plasmid. He also ligated the two pieces of DNA together and transformed into DH5α *E. coli* cells. Silva prepared a glycerol stock of these cells to store in the -80°C freezer (labeled SDB10).

A diagnostic analysis was run to ensure that the ligation step was successful. SDB10 cells were grown on LB-amp plates. One colony was inoculated into 5 mL liquid LB-amp media. This plasmid was purified using QIAPrep Spin Miniprep Kit (QIAGEN) and underwent double digestion using *Sac*I and *Xho*HI restriction endonucleases from New England Biolabs. The digestion products were run on an agarose gel to visualize the size of DNA fragments present.

The gene product was transformed into *S. cerevisiae* and selected for using the same methods as used for the lower expression plasmid, and finally, struck onto the 5-FOA drug plate. Positive controls for all SD-Ura/-LEU2 and SD-LEU2 plates used *S. cerevisiae* transformed with an empty 2μ plasmid and no eEF3. On the 5-FOA plate, *S.* cerevisiae expressing *S. pombe* eEF3 was again used as the positive control. A negative control using untransformed *S. cerevisiae* was also tested on the 5-FOA plates.

3. RESULTS

3.1 Low-Expression Plasmid Results

To begin this investigation of eEF3 functional conservation, the eEF3 gene was first isolated from *C. reinhardtii*, and a low expression plasmid vector was created. The CrEF3 gene to be used was extracted from its genomic library and amplified using a PCR reaction (data not shown). Double digestion reactions were performed using the *BamI* and *Xho*I enzymes, allowing the CrEF3 gene product to be prepared for ligation. The digested CrEF3 insert can be seen in the clean band in the 3.2 kb band (Figure 7A). The *pTK328* plasmid to be used was also successfully digested and purified, as seen in the clean, single band at 7.5 kb (Figure 7B).

Gene selection and transformation were successful. The plasmid was ligated and transformed into the DH5α *E. coli* cells, as indicated by plate growth (Table 8). The four colonies that grew indicated that the cells contained a ligated plasmid and were expressing the ampicillin-resistance (AmpR) gene. Based on a diagnostic double digestion and agarose gel analysis of the plasmid present within the cells, the gene successfully inserted into the plasmid and was expressed in two of the *E. coli* colonies, numbered Colonies 1 and 4 in Figure 8. The ligated LE-CrEF3 plasmid was purified from cultures that had been inoculated with Colonies 1 and 4; these two bacterial cultures containing LE-CrEF3 were renamed KBB1-1 and KBB1-2.

This plasmid construct was successfully transformed into the *S. cerevisiae* strain. These markers are present on the LE-CrEF3 and Ex-ScEF3 plasmids, respectively.

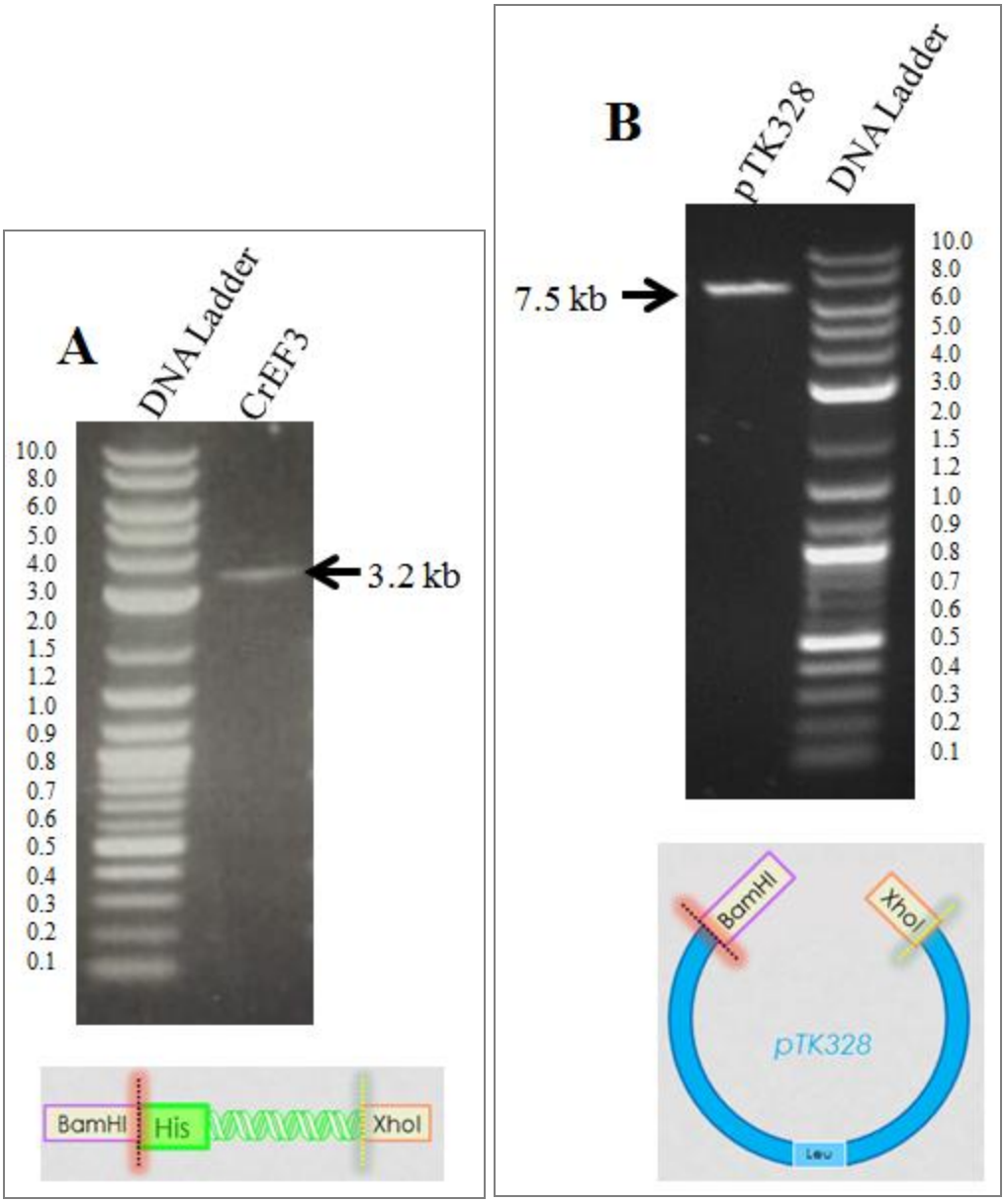

Figure 7 – Agarose analysis of purified CEN plasmid and gene insert. The gene products from the double digestion reactions of both the *pTK328* plasmid and the CrEF3 were successfully purified. (A) The purified CrEF3 gene product is shown at 3.2 kb, and (B) the purified *pTK328* plasmid product is shown at 7.5 kb.

Table 8 – Plate counts for DH5α Transformants Containing LE-CrEF3

Reaction #	DNA Used	Volume Plated	Colonies Present
1	Ligation product	50.0	4
		200.0	1
2	Ligation product	50.0	0
		200.0	0
3	pUC19	10.0 (diluted in an additional 90.0 µL liquid LB-amp media)	TNTC
		100.0 (undiluted)	TNTC
4	Uncut pTK328 vector DNA	10.0 (diluted in an additional 90.0 µL liquid LB-amp media)	TNTC
		100.0 (undiluted)	TNTC

*__Note__: TNTC indicates that there were >300 colonies present.

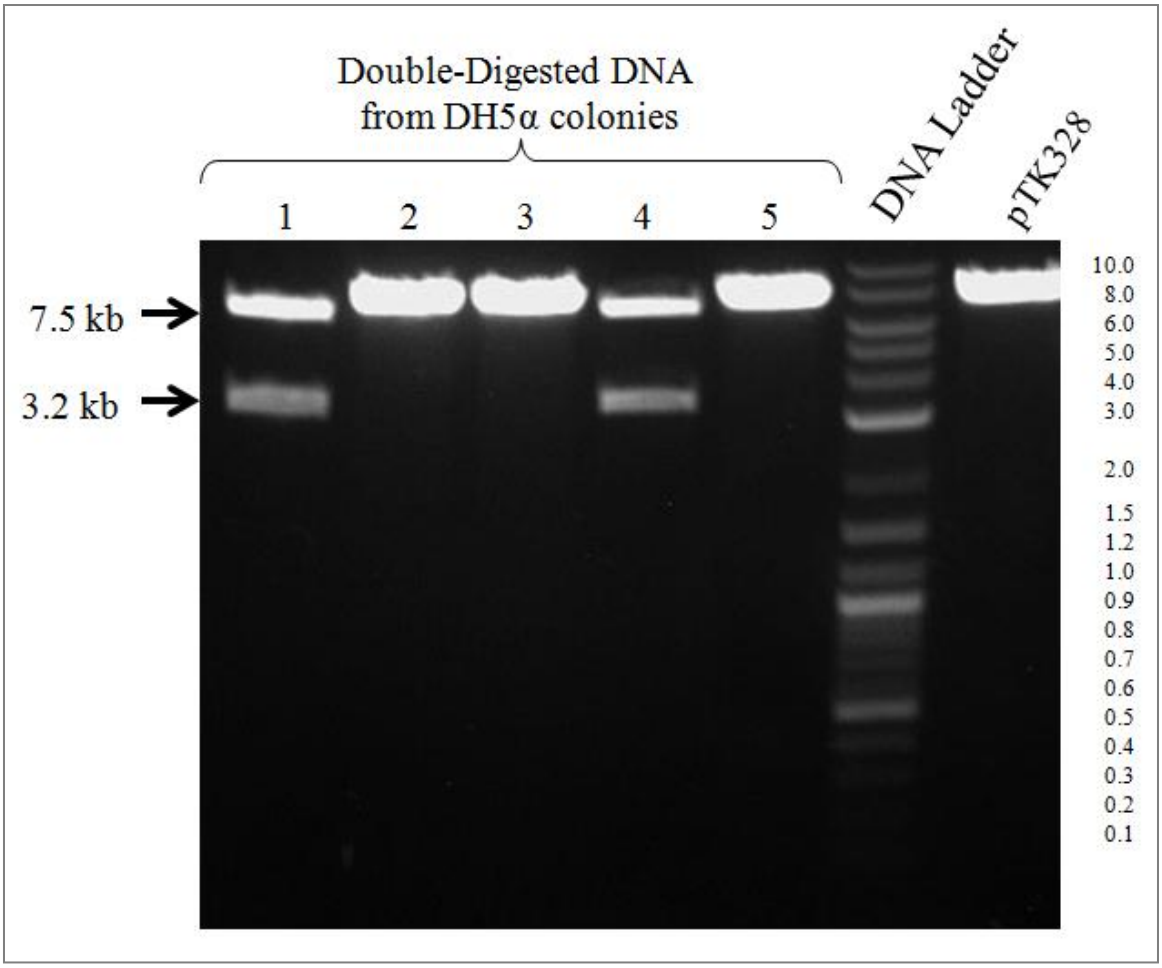

Figure 8 – Verification of successful ligation using low expression plasmid. Extracted DNA from DH5α colonies numbered 1 and 4 shows that both the *pTK328* plasmid and the *C. reinhardtii* gene fragments are present at 7.5 kb and 3.2 kb following a diagnostic double-digestion reaction using *Bam*HI and *Xho*I. The *E. coli* strains containing colonies 1 and 4 were labeled KBB1-2 and KBB1-2, respectively.

Experimental transformants containing LE-CrEF3 from KBB1-1 and KBB1-2 showed full growth on the initial SD-Leu/-Ura plates, as did the positive control transformant containing pTK328 (data not shown). Cells are unable to grow on this plate if they lack the LEU2 and URA3 growth selection markers. The media was shown to be successfully formulated by the lack of growth by the negative control containing untransformed *S. cerevisiae* cells that had only the Ura-marked plasmid. Experimental and positive control transformant cultures also grew successfully when re-struck onto SD-Leu media (Figure 9).

As seen in Figure 10, the 5'-FOA drug plates allowed growth only of the positive control; the negative control and the experimentally transformed strains did not grow on this drug-selection media. Positive control used transformants known to express SpEF3 present on a Leu-marked plasmid, and thus they did not rely on their Ura-marked Ex-ScEF3 plasmid and were able to grow, as expected. The negative control used untransformed *S. cerevisiae* that required expression of the Ex-ScEF3 plasmid, thereby poisoning the cells with the toxic product. For cultures that did not grow up into fully developed colonies, tiny pinpoint colonies were present, indicating initially viable cells were killed after being subjected to the toxic product formed by the 5'-FOA. These pinpoint colonies formed within the first twelve hours yet were prohibited from growing into a full colony due to the 5'-FOA drug present in the growth media.

Several western blots were performed to visualize the protein expression levels of the transformed *S. cerevisiae* cells. Two blots were analyzed (Figure 11). One used a primary antibody against the histidine tag present on the CrEF3 gene used. A second blot

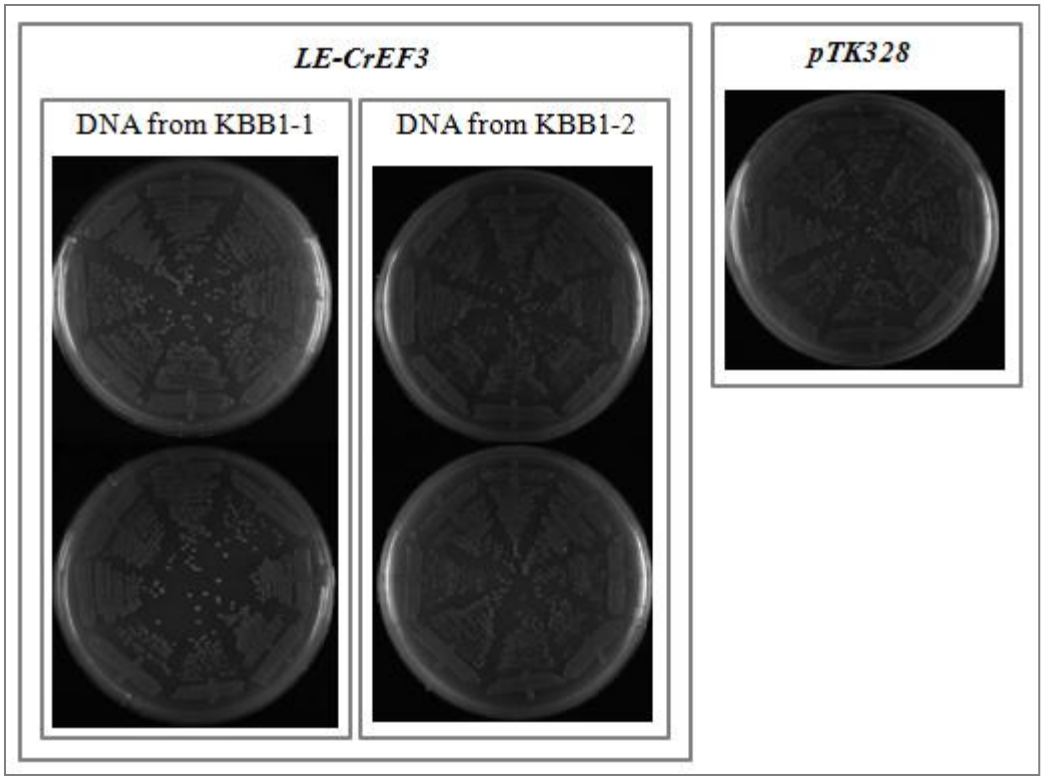

Figure 9 – Growth on SD-Leu selection plates using eEF3 ligated within a low expression plasmid. All cultures containing the Leu-marked plasmid showed plate growth, indicating that transformation was successful. Plates are labeled by the DNA used to transform *S. cerevisiae*. When re-streaking from previous plates, one colony yields one "wedge;" a total of eight yeast clones were tested on each plate.

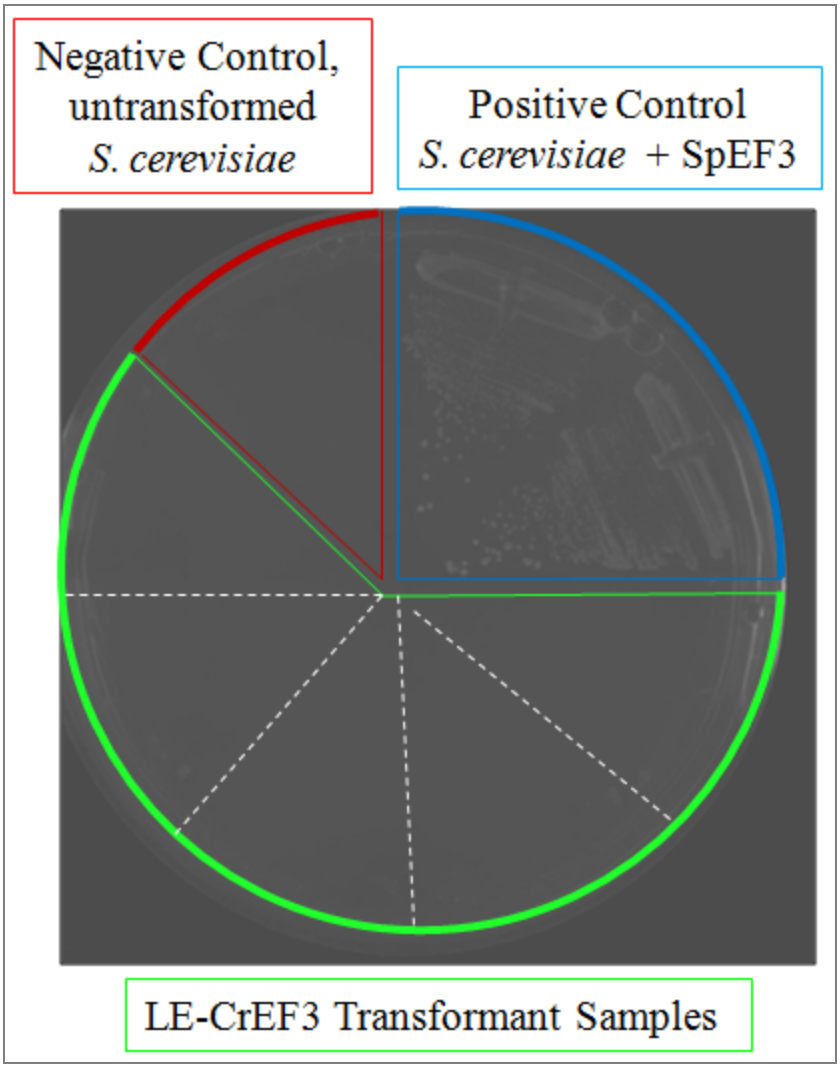

Figure 10 – Growth on the 5'-FOA drug plate using CEN plasmid. Positive controls and negative controls were successful, using *S. cerevisiae* expressing SpEF3 and untransformed *S. cerevisiae*, respectively, as seen by the growth within the wedges outlined in blue (positive control) and the lack of growth within the wedge outlined in red (negative control). However, there was no growth from any of the samples tested, as seen in the wedges outlined in green.

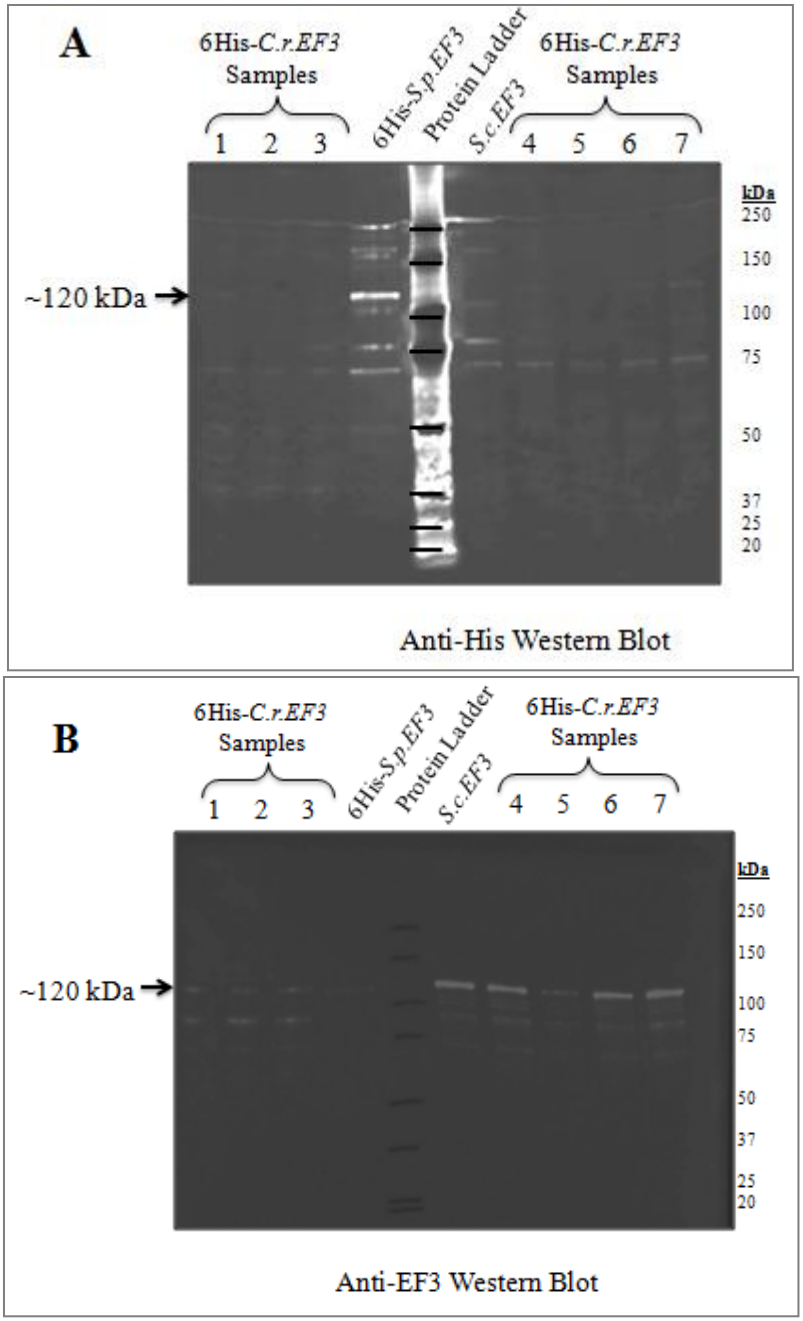

Figure 11 - Western blot investigation of eEF3 protein expression levels using CEN plasmid. These western blots show low CrEF3 expression levels by *S. cerevisiae*. The ladder used has bands at 250, 150, 100, 75, and 50 kD. (A) In the anti-His western blot, the His-tagged *ChlEF3* protein was lightly expressed and present at about 120 kDa. (B) In the anti-EF3 western blot, eEF3 expression was present but highly variable.

monitored the expression levels of the CrEF3 protein itself. A positive control and negative control used yielded the expected results; positive control colonies demonstrated a bright band at 120 kDa, which corresponds to the protein size of eEF3, while the negative control lacked a Histidine-tagged protein of this size (Figure 11 A and B). Faint expression of the histidine-tag protein was observed using the anti-histidine in Figure 11A, and expression levels of the all eEF3 protein in the cells varied, based on the anti-eEF3 antibody in Figure 11B.

3.2 High-Expression Plasmid Results

The experiment was repeated with a high-expression plasmid to determine whether the inability of CrEF3 to maintain *S. cerevisiae* viability resulted from limited availability of the protein, due to an insufficient copy number of CrEF3 present in the cell when under the expression levels of a CEN plasmid. The high-expression plasmid (HE-CrEF3) used contained the 2-micron (2μ) promoter to increase the copy number of CrEF3 in the *S. cerevisiae* cells.

When repeating the methods using the 2μ plasmid in place of the CEN plasmid, similar results were observed. The gene and 2μ plasmid were both successfully digested using *Sac*I and *Xho*I (Figure 12). The CrEF3 gene successfully ligated into the 2μ plasmid and transformed into the DH5α *E. coli* competent cells, as verified by agarose gel analysis (Figure 13). The amplified plasmid was isolated and used to transform *S. cerevisiae* cells. These new transformant cells containing the CrEF3 gene grew successfully upon both the initial transformation on SD-Ura/-LEU2 plates and the re-struck SD-LEU2 plates (Figure 14). The final test of growth on a 5-FOA drug plate showed limited positive control growth and no transformant growth (Figure 15).

The protein expression levels of the eEF3 protein were analyzed in the transformant *S. cerevisiae* cells used for the positive control, negative control, and experimentally transformed cell lines using a western blot and a primary antibody against eEF3 (Figure 16). The expression levels of the eEF3 protein varied. Low expression was

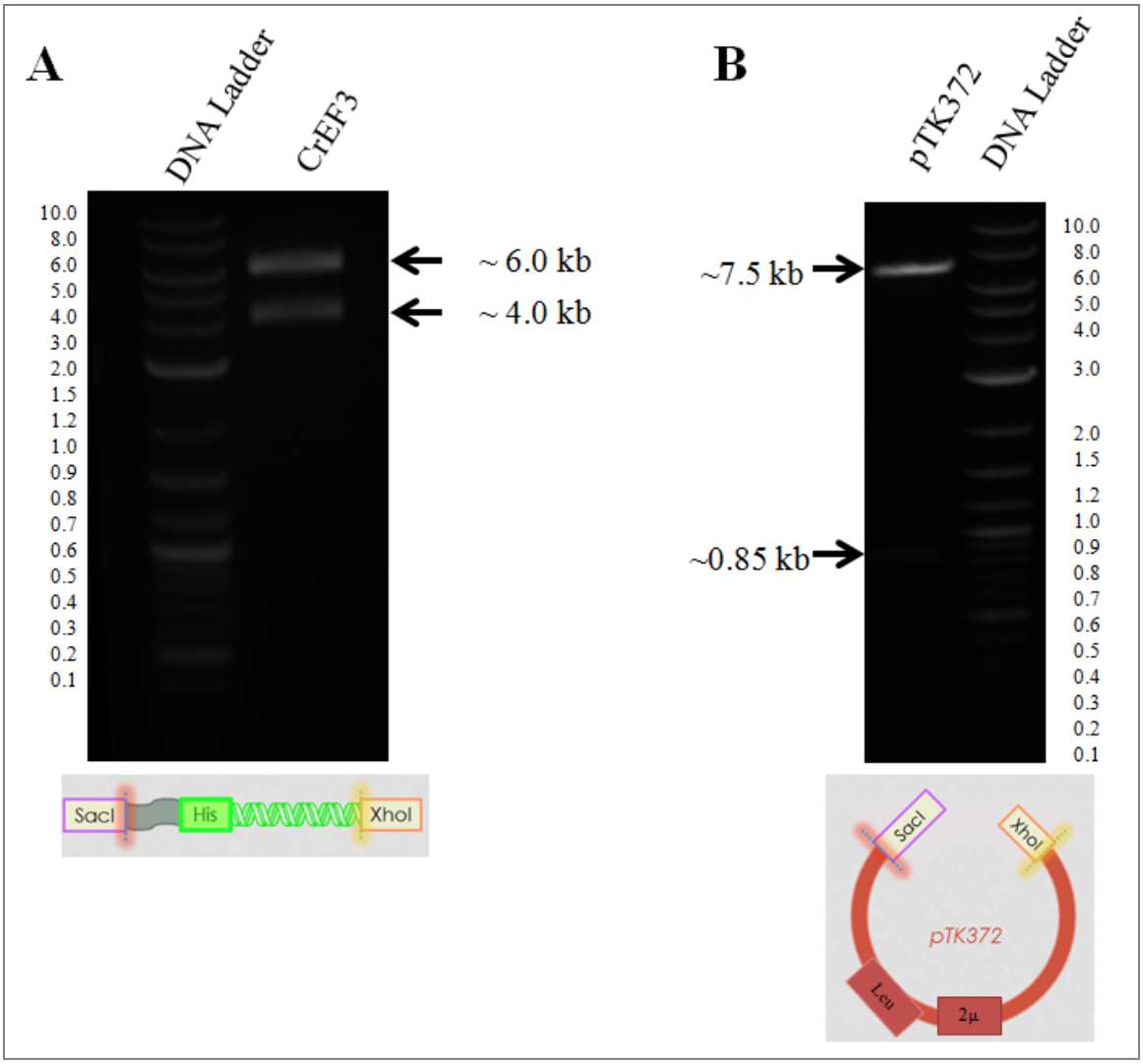

Figure 12 – Agarose analysis of digested CrEF3 and high expression plasmid. The gene products from the double digestion reactions of both the high expression pTK372 plasmid and the LE-CrEF3 were ran on agarose gels. (A) The CrEF3 digestion product from LE-CrEF3 is shown at 4.0 kb. The pTK328 plasmid is also present at about 6.0 kb. (B) The pTK372 digestion product is shown at 7.5 kb, with the excised band from pTK372 shown at 0.85 kb.

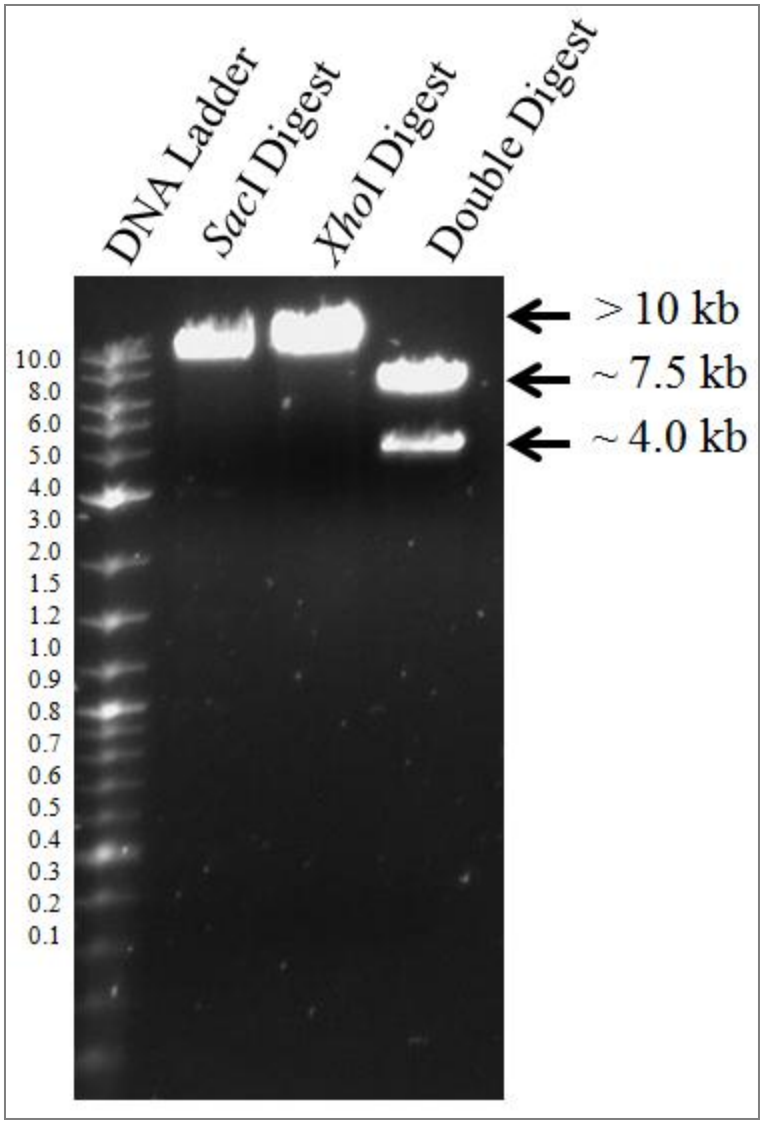

Figure 13 – Verification of successful ligation of HE-CrEF3. Extracted DNA from DH5α colonies shows that both the *pTK372* plasmid and the *C. reinhardtii* gene fragments are present and ligated following a diagnostic digestion reaction. Single digests were run using *Sac*I and *Xho*I independently, and a double digest was run using both enzyme reagents. The CrEF3 is present at about 4.0 kb, and the high expression *pTK372* plasmid is present at about 7.0 kb. The CrEF3 band size increased from its previous 3.2 kb as a result of the change in restriction enzyme from *Bam*HI to *Sac*I, which included the 670 bp pGAL1 gene sequence in the second CrEF3 transformation reaction.

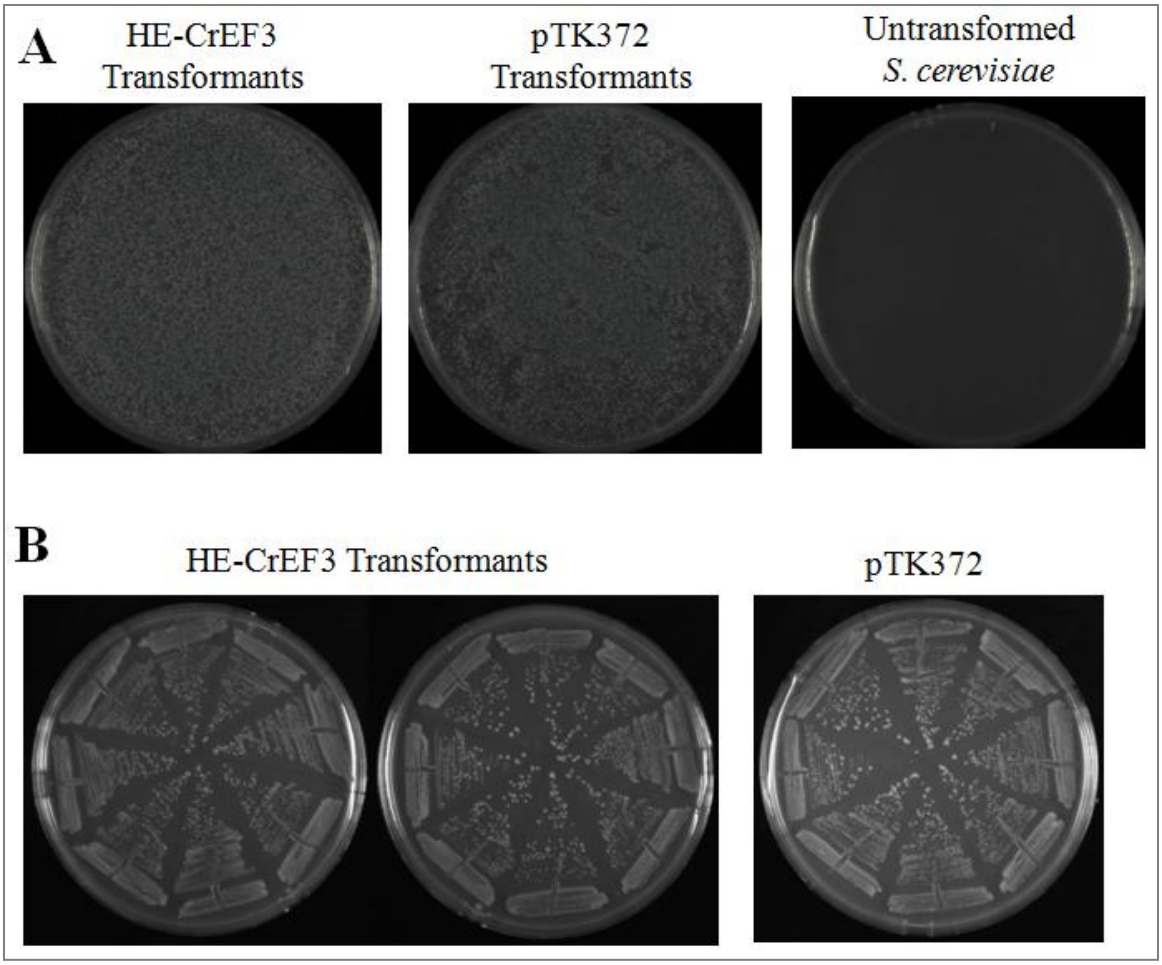

Figure 14 – Growth on selection plates using a high-expression plasmid. (A) Initial transformant cells plated on SD-Ura/-Leu plates was successful. The positive control pTK372 transformants, which contained the high expression plasmid with the Leu-marker yet lacking CrEF3, showed growth. The negative control, untransformed *S. cerevisiae* lacking the Leu-marker, did not grow on SD-Leu plates. (B) Transformant cells re-streaked onto SD-Leu plates. All plates showed growth, indicating that transformation was successful.

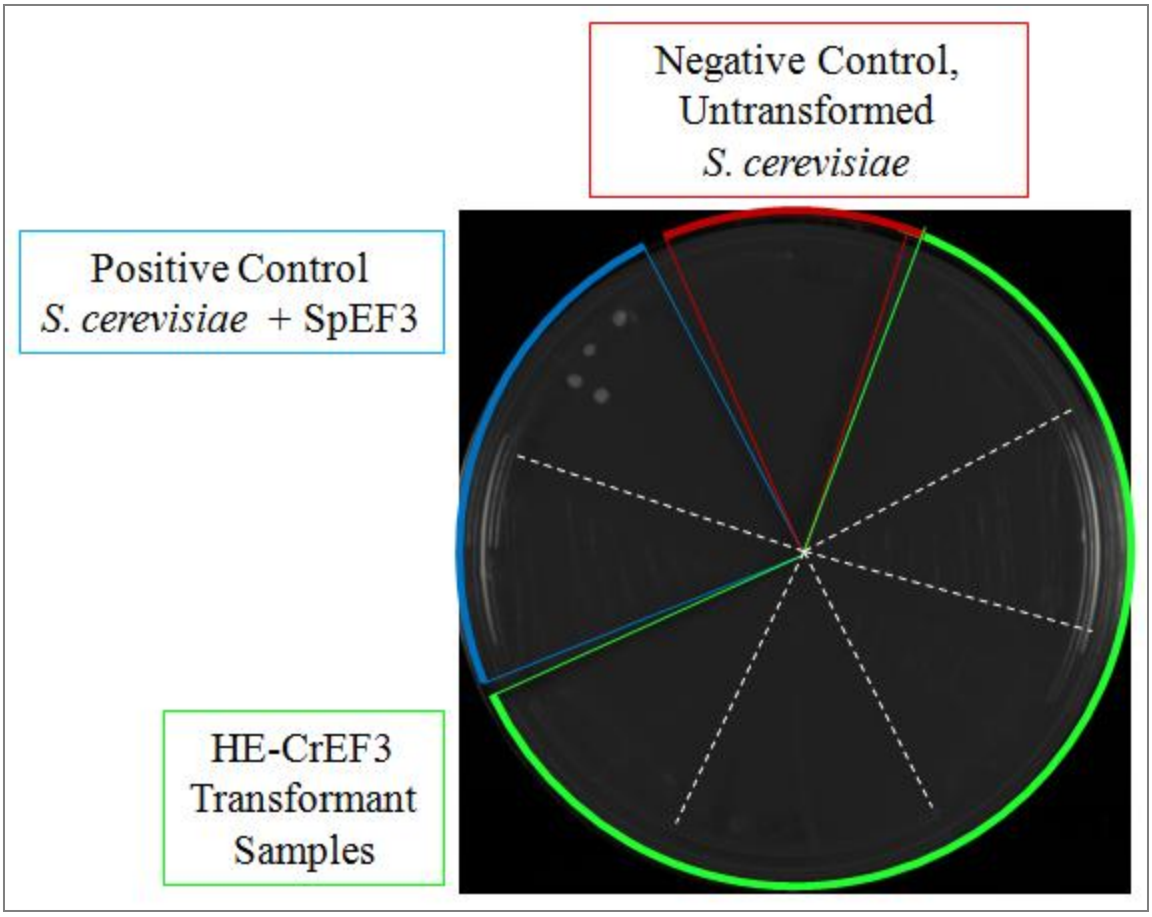

Figure 15 – Growth on the 5'-FOA drug plate using 2µ plasmid. Positive controls and negative controls were successful, using *S. cerevisiae* expressing SpEF3 and untransformed *S. cerevisiae*, respectively, as seen by the growth within the wedges outlined in blue (positive control) and the lack of growth within the wedge outlined in red (negative control). However, minimal positive control growth was noted, and there was no growth from any of the samples tested, as seen in the wedges outlined in green.

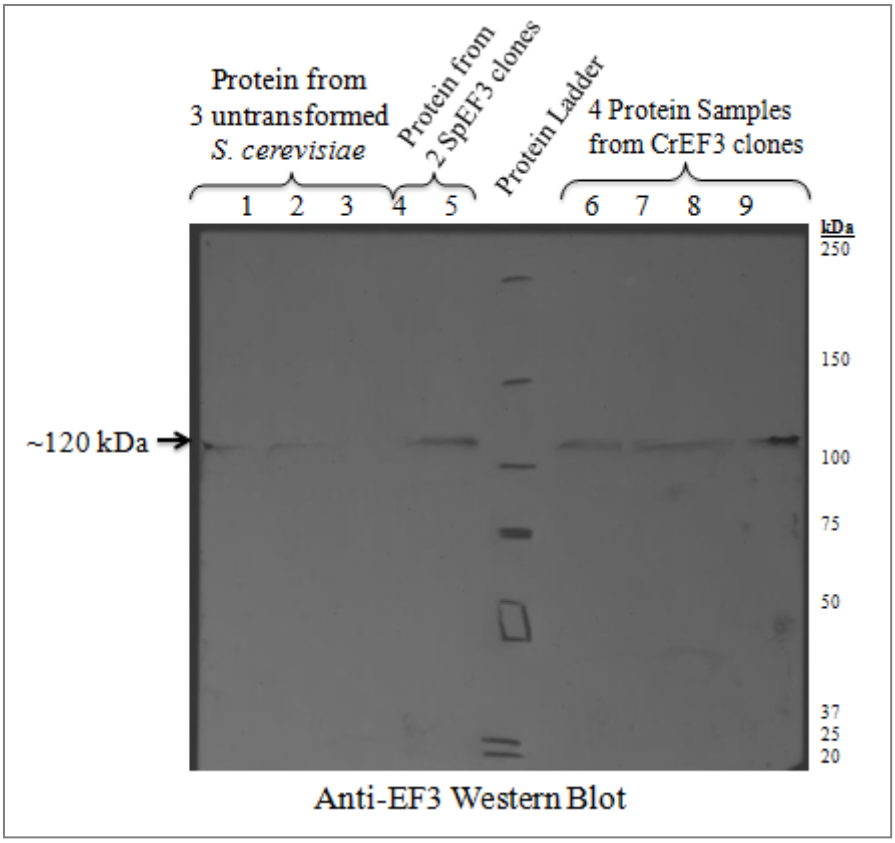

Figure 16 – Western blot investigation of eEF3 protein expression levels using 2µ plasmid. eEF3 expression was variable, as seen at the indicated band size of about 120 kDa.

observed in the negative control that used untransformed *S. cerevisiae*. The positive control *S. cerevisiae* cells containing *S. pombe* eEF3 had high expression levels. Expression of the eEF3 protein in transformant colony samples tested varied.

4. DISCUSSION – ANALYSIS OF eEF3 FUNCTIONAL CONSERVATION

There has been a recent surge in the incidence of fungal infections (McNeil et al. 2001; Martin et al. 2003; Mean et al. 2008). Unfortunately, existing treatments come with high toxicity and are rarely able to cure fungal infections (Enoch et al. 2006; Groll and Walsh 2002; Marr 2004; Pfaller and Diekema 2010). Clearly, there is a need for an improved method of clinical treatment with high efficacy and low toxicity. One conceived method is to devise a targeted drug therapy that can selectively inhibit fungal growth, without harming human cells. This novel method would improve the efficacy of anti-fungal drug treatment. eEF3 is a potential anti-fungal drug target due to the fact that it is essential for lower-eukaryotes such as fungi, yet it is completely absent from mammalian cells. This project sought to determine the extent to which eEF3 is functionally conserved within lower eukaryotes by performing a plasmid shuffle technique to clone eEF3 from *C. reinhardtii* into *S. cerevisiae*. This scheme provides important insight on the conservation of the protein since these two organisms are more divergent that those that have been used in the past to analyze eEF3 functional conservation. In order to be a viable drug target, eEF3 should ideally be highly conserved among those organisms that rely on it to survive.

The hypothesized result is that CrEF3 will successfully complement and support viable *S. cerevisiae* cell growth. Based on previous studies using eEF3 from *C. neoformans* and *S. pombe*, it is expected that growth rates could slow and the average cellular sizes could shrink in transformant *S. cerevisiae* cultures, relative to the increased taxonomic divergence of the two species being complemented (Blakely et al. 2001;

Colmer 2013). In particular, due to the fact that the protein alignment of ScEF3 versus both CrEF3 and *C. neoformans* both show 46% identity (BLAST - NCBI), it was expected that CrEF3 would be able to support viable *S. cerevisiae* growth to the same extent. If this is the case, it would indicate that the protein is moderately conserved functionally. This specificity and high conservation would make eEF3 a strong candidate for a molecular target.

To test the functional conservation of eEF3 between *C. reinhardtii* and *S. cerevisiae*, two tests of gene expression and function were run using the same protocol with two plasmids of different expression levels.

4.1 Low-Expression Plasmid Experiment

To begin the investigation, the complete CrEF3 gene was first extracted and amplified. The first test used the same low-expression CEN plasmid that had been previously used to successfully transform SpEF3 into *S. cerevisiae* (Colmer 2013). The eEF3 gene extract and a CEN plasmid were both individually digested using the same enzymes to allow complementary ligation. Successfully ligated gene products were identified using DH5α competent *E. coli* cells, isolated, and transformed into *S. cerevisiae* (Figure 9). After a series of growth selection that encouraged *S. cerevisiae* to express and utilize the CrEF3 gene rather than the exogenous ScEF3, select transformant colonies were struck onto 5-FOA drug plates to determine whether CrEF3 could support cell viability or if cells retained the Ura-marked ScEF3. The colonies from the same cultures that had been tested on 5-FOA plates were grown up in liquid SD-LEU2 media and lysed to extract cytoplasmic proteins. Western blot analysis was used to determine

the level of CrEF3 expression from the low-expression plasmid.

The CrEF3 gene was pre-tagged with a six-histidine residue to allow easy identification. In total, it was known to be approximately 3.2 kb in size. Initial PCR tests run by the Kinzy laboratory successfully amplified this gene of interest using the forward and reverse primers selected. This was confirmed using an agarose gel electrophoresis diagnostic of a small amount of PCR product (data not shown). The positive and negative controls for this step were successful, ensuring that the band present was, in fact, representative of the CrEF3 gene desired and not merely a contaminant.

In order to transform the gene into *S. cerevisiae*, a plasmid needed to be constructed. A low-expression CEN plasmid, pTK328, had known success with the transformation protocol used (Colmer 2013).

The double-digestion process aided in the purification process of the gene, as seen by the clean bands for both the plasmid and gene insert in Figure 7. The digestion products were easily extracted from the agarose gel and successfully purified.

Ligation was conducted in a simple reaction mixture. To ensure the gene inserted successfully, and to amplify the plasmid construct product, the ligation product was transformed into DH5α *E. coli* competent cells. The resultant colonies that grew on LB-Amp plates were cultured in liquid media and lysed to extract plasmid DNA. A diagnostic double digest confirmed that two colonies contained the gene insert, indicated by the fact that there were two bands present: the plasmid at 7.5 kb and the insert at 3.5 kb (Figure 8). The other three colonies shown have a molecular weight of 7.5 kb and are

much more intense. The absence of a band at 3.5 kb shows that no eEF3 was inserted and only the low-expression plasmid vector was present. Additionally, the higher intensity shows that the there was more gene product within the equal volume loaded into the gel. This indicates that the *E. coli* cells were able to replicate the empty plasmid more rapidly than the plasmid containing the eEF3 gene, due to its smaller plasmid size of 7.5 kb total versus 11.0 kb total. This could be explained by the fact that the eEF3 gene adds an additional 3.5 kb in size to the plasmid, which is almost a 50% increase in nucleotides to be replicated. This extends the length of time that the cells require to replicate the entire plasmid. Therefore, although the cultures grew for the same amount of time, those cultures containing plasmids with the eEF3 insert contained fewer plasmid copies per cell than those without the insert.

The purified LE-CrEF3 plasmids were used to transform *S. cerevisiae*. Based on the initial transformation plates, which were grown on SD-Ura/-Leu media, the transformation was successful. Negative control *S. cerevisiae* that underwent the transformation protocol without LE-CrEF3 did not grow, which was expected since they lacked the LEU2 gene present on the CEN plasmid (data not shown). Positive control *S. cerevisiae* that had been transformed with an empty CEN plasmid was able to grow since they contained the LEU2 marker. Both experimental plates tested were able to grow, since they contained both the URA3 marker from the exogenous copy of ScEF3 as well as the LEU2 marker from the successful transformation of the CrEF3 gene. This result was expected for successful transformant cells, which were encouraged to express both plasmids in order to obtain the uracil and leucine nutrients necessary for growth.

Transformed *S. cerevisiae* cells containing the CrEF3 gene and transformed positive control *S. cerevisiae* cells were both re-streaked on SD-LEU2 plates in an attempt to drive cells to express CrEF3. For experimental cells, this process was used to provide selective pressures for *S. cerevisiae* cells to express and utilize CrEF3 preferentially over the exogenous ScEF3 gene present, since expression of the LEU2 marker was necessary to create leucine, whereas the uracil present in the growth media negated the need to express the URA3 marker present on the plasmid containing ScEF3. Transcription of the LEU2 marker leads to increased expression of CrEF3, since the polymerase may continue transcribing around the plasmid. Further, the transcription of LEU2 recruits the necessary proteins for expression of the other genes on the plasmid and exposes the promoter region of the CrEF3 gene. If CrEF3 was able to support viable cell growth, cells may even kick out the plasmid containing the ScEF3 copy. All transformant cultures grown on SD-LEU2 grew, but at this point, it was not conclusive whether or not the cells were relying upon CrEF3 at all, or if CrEF3 was being used preferentially over ScEF3.

The final test was the 5-FOA drug plate. If the transformed *S. cerevisiae* cells retained their own copy of eEF3 on the URA3-marked plasmid, the cells would not be able to grow since the expressed Uracil gene and its resultant protein product would poison the cell. The plates were proven to be correctly formulated, based on the lack of growth by untransformed *S. cerevisiae* containing the URA3-marked plasmid as well as the prolific growth of *S. cerevisiae* cells expressing *S. pombe* eEF3 that were known to have kicked out the exogenous URA3-marked plasmid containing ScEF3 (Figure 10).

The transformant cells containing CrEF3 did not grow on the 5-FOA drug plates (Figure 10). They had retained the *S. cerevisiae* copy of eEF3, and even in a detrimental environment, preferentially relied upon their own copy of the protein rather than the *C. reinhardtii* copy. This shows that the EF3 protein is not functionally conserved between these two species. This may have been caused by several factors. Perhaps the protein had structural or functional differences that did not permit the *C. reinhardtii* protein to perform its role within *S. cerevisiae* cells, as a result of the low homology between the EF3 proteins of these two species (42.5% identity– see Table 1). Perhaps the number of physical copies within the cell was too low to allow expressed CrEF3 to sustain growth and stay up-to-pace with the translational needs of the cells.

To further elucidate the expression levels of cytoplasmic proteins, two western blots were conducted. One analyzed the expression level of the 6-histidine tag within the cell in order to gain insight on the expression levels of the histidine-tagged CrEF3. A clear band is present within the positive control at about 120 kDa, the appropriate band for the eEF3 protein. The negative control shows that there is no band of this size. Expression was variable within sample *S. cerevisiae* transformants, with only faint bands appearing at the 120 kDa band. This showed that there was very low expression of the CrEF3 protein.

To confirm this conclusion, a second western blot was run using a primary antibody against eEF3 itself. Considering that eEF3 was present in all cells from which the proteins were extracted, the expression may have been coming from either the ScEF3 copy or from the CrEF3, or from a combination of both. This western blot showed that

there was highly variable eEF3 expression (Figure 11). The SpEF3 clones had a very faintly visible band present at about 120 kDa, whereas the untransformed *S. cerevisiae* showed a very bright band at 120 kDa. Since equal concentrations and volumes of whole cell protein was loaded, this shows that there is a different binding affinity of the anti-EF3 antibody for the protein copy from *S. cerevisiae* and *S. pombe*. This western blot showed that the expression of the transformed eEF3 that had successfully grown on 5-FOA when *S. cerevisiae* utilized *S. pombe* eEF3 was much lower than the expression of the native *S. cerevisiae* copy.

This difference in antibody binding affinity makes it likely that there would also be a difference in binding affinity between the binding of anti-EF3 antibody and either ScEF3 or CrEF3. It appears that there was a mix of expression among samples containing the CrEF3. It appears that the first three samples and the fifth sample had limited expression, perhaps due to a diminished reliance upon ScEF3. Samples four, six, and seven had much higher levels of eEF3 expression, comparable to that of the untransformed *S. cerevisiae*, perhaps due to a continued reliance upon the exogenous ScEF3.

4.2 High-Expression Plasmid Experiment

After discovering that LE-CrEF3 was unable to sustain viable *S. cerevisiae* cell growth, a second experiment was designed to boost expression of CrEF3. Potentially, a higher copy number of CrEF3 would enable CrEF3 to sustain viable cell growth in place of the exogenous ScEF3 protein. This sought to investigate whether the failure to support viable growth was a result of insufficient eEF3 protein available within the cell to

perform its transformation role. This new design increases the likelihood that sufficient CrEF3 protein would be present to meet the translational needs of the cell.

CrEF3 was successfully isolated from LE-CrEF3 using *Sac*I and *Xho*I, yielding a larger gene fragment as a result of the inclusion of the 670 bp pGAL1 gene from the low expression plasmid, which falls between the binding sites of the restriction enzymes (Figure 6). A diagnostic test also verified that these restriction enzymes were active for the new high expression plasmid. It is apparent that both enzymes were effective, based on the presence of two bands, which tells that the plasmid was not merely linearized but was actually sliced into two fragments. The plasmid vector and gene insert were both prepared for ligation by the creation of complementary sticky ends. Importantly, if ligation were to occur between the newly created sticky ends, without the insertion of the gene, the ligation site would be destroyed. This would block the CrEF3 gene from gaining access to the plasmid for insertion. Fortunately, as indicated in Figure 13, successful ligation took place between the CrEF3 gene extracted from LE-CrEF3 and the new high-expression plasmid, to yield HE-CrEF3.

HE-CrEF3 was extracted from the DH5α transformant cells was transformed into *S. cerevisiae* (Figure 14). The negative control, which went through the transformation process with dd-H_2O in place of DNA, showed no growth on the SD-Ura/-Leu plates. This was expected, since this control lacks the LEU2 marker needed to grow on these plates; only the URA3 marker was present on the exogenous ScEF3 plasmid. The positive showed the expected growth. This was designed to contain an empty high-expression plasmid, thus providing the LEU2 marker and the exogenous URA3 marker,

both of which are required for growth. Importantly, the positive control excludes the CrEF3 gene, as this may impact cell growth. This allows confirmation of a successful experimental design, while excluding any variable that may be introduced by the addition of the CrEF3 protein.

The experimental *S. cerevisiae* transformants also showed the desired growth on the initial transformation plates. However, at this step, it was unclear which copy of eEF3 was supporting viable cell growth. For this reason, qualitative analysis of the ability of CrEF3 to support viable *S. cerevisiae* growth was performed identically to the previous round of experiments. Growth selection SD-Leu plates were inoculated with transformant cells to promote expression of the HE-CrEF3 plasmid. All of these plates showed full, uninhibited growth (Figure 14).

An absolute test of the ability of HE-CrEF3 was performed using the same 5-FOA drug plate as above. However, once again, no growth was observed past the initial pinpoint colonies, indicating that CrEF3 was unable to support viable *S. cerevisiae* growth (Figure 15). It must be noted that the positive and negative controls were successful, as indicated by the weak growth of the positive control, which used *S. cerevisiae* tranformants containing SpEF3, and lack of growth of the negative control, which used untransformed *S. cerevisiae*. Importantly, limited positive control growth was observed, as noted by the fact that only four colonies were present, as opposed to the expected growth throughout the full wedge. A separate test was performed, and it was concluded that this was the result of the glycerol stock for these transformant cells having over-thawed, causing the cells to settle, and thus decreasing the number of cells

inoculated. The positive controls for the 5-FOA plates were inoculated directly from these glycerol stocks, and thus fewer cells were picked up than expected. The limited growth is not caused by any error in the 5-FOA plate composition.

Once again, the expression levels of CrEF3 needed to be analyzed in greater detail through a western blot analysis. An anti-EF3 western blot showed that expression levels of eEF3 varied within samples (Figure 16). Three of the samples loaded originated from cultures expressing ScEF3 only, using one protein sample isolated from untransformed *S. cerevisiae* and two protein samples from *S. cerevisiae* transformed with an empty high expression plasmid. Two transformant samples were also loaded, using purified protein from cells expressing SpEF3. These transformant samples showed darker bands at 120 kDa, indicating that the transformant eEF3 was expressed at a higher level than the exogenous ScEF3. From this, it can be seen that more EF3 is needed to support viability in a transformed cell, whereas the cells are more efficient at using their own copy of Sc EF3 and therefore do not require as much ScEF3 present.

Three of the samples tested showed similar expression levels to the untransformed *S. cerevisiae* expressing ScEF3 only. Notably, for equal concentrations of protein loaded, a significantly higher level of expression was seen in sample nine (Figure 16). This could be the result of combined expression from both ScEF3 and CrEF3 within the cell; however, based on the results of the 5-FOA plates, this increased expression was not sufficient to support viable cell growth, perhaps due to significant structural differences in the protein.

4.3 Future Research Directions

To complete the second goal of analyzing high-expression transformants, an anti-His western blot should be run on the proteins from the HE-CrEF3 transformants to quantify how much EF3 expression originates from the CrEF3 compared to how much comes from ScEF3. This would finalize results from the high-expression plasmid results.

Further literature research determined that the failure of CrEF3 to support viable *S. cerevisiae* cells may be the result of alternate codon usage. *C. reinhardtii* translates codons into amino acids based on a slightly different code than *S. cerevisiae* does (Elzanowski and Ostell 2013; Nakamura 2007). Therefore, when translating the same gene sequence, *S. cerevisiae* creates a different protein than *C. reinhardtii* would. The extent of structural changes caused by expressing CrEF3 using *S. cerevisiae* expression machinery is determined by whether the alternate codons used by these two species result in codons for the same, similar, or different amino acid residues, based on the amino acid side chain present.

For the purposes of this experiment, this difference could be corrected by introducing substitutions in the gene sequence to artificially convert the gene to the "proper" gene sequence, so that the CrEF3 protein may be "properly" translated by *S. cerevisiae*. A new transformation should be conducted using a modified CrEF3 gene sequence that may be expressed by *S. cerevisiae* in the same way that it is naturally expressed in *C. reinhardtii*. This would elucidate whether eEF3 is, in fact, highly conserved, and whether the decreased expression levels seen in *S. cerevisiae* expressing increasingly divergent copies of eEF3 is merely caused by differences in the reading ability of the translation proteins of the two organisms being investigated.

Further questions may also be investigated regarding eEF3. If the protein proves to not be conserved between *C. reinhardtii* and *S. cerevisiae* following the results of the alternate codon experiment, another functional complementation experiment could be conducted to clone eEF3 between *S. cerevisiae* and a fungal organism from a different phyla than has been studied in the past. In particular, species from the Chromista and Zygomycota phyla should be investigated next, as these phyla contain pathogenic fungi. This would determine the degree of conservation among clinically relevant fungi.

What protein or set of proteins serve this function in upper eukaryotes? A separate investigation could be conducted to determine the thermodynamic and kinetic benefits that upper eukaryotes have gained by losing this protein.

5. CONCLUSION

This experiment has demonstrated that eEF3 is less functionally conserved among lower eukaryotes than hypothesized. Due to the successful functional complementation of eEF3 among fungi from the two different phyla Basidiomycota and Ascomycota, it was predicted that the protein would successfully complement between the non-fungal lower eukaryote *C. reinhardtii* and *S. cerevisiae*, the model organism that has been used in the past for these conservation studies. eEF3 was not able to support viable cell growth when transformed between taxonomically divergent *C. reinhardtii* and *S. cerevisiae*. Increasing the copy number of CrEF3 being expressed in *S. cerevisiae* also failed to support functional complementation. This may be the result of structural differences in the eEF3 protein of the two species. Another possible cause for the lack of functional conservation between these two species investigated is the presence of alternate codon usage between *C. reinhardtii* and *S. cerevisiae*. Because of this, the same genetic sequence may be converted into a different amino acid sequence, producing a different protein in *S. cerevisiae* than what is actually produced in *C. reinhardtii*.

LIST OF TABLES AND FIGURES

Tables

Figures

LITERATURE CITED

Anand M, Balar B, Ulloque R, Gross ST, Kinzy TG. 2006. Domain and nucleotide dependence of the interaction between *Saccharomyces cerevisiae* translation elongation factors 3 and 1A. The Journal of Biological Chemistry 281(43): 32318-32326.

Anand M, Chakraburtty K, Marton MJ, Hinnebusch AG, Kinzy TG. 2003. Functional Interactions between Yeast Translation Eukaryotic Elongation Factor (eEF) 1A and eEF3. The Journal of Biological Chemistry 278(9): 6985-6991.

Andersen CBF, Becker T, Blau M, et. al. 2006. Structure of eEF3 and the mechanism of transfer RNA release from the E-site. Nature 443(12): 663-668.

Andersen CF, Anand M, Boesen T, Van LB, Kinzy TG, Andersen GR. 2004. Purification and crystallization of the yeast translation elongation factor eEF3.

Arunaloke C. 2007. Epidemiology of central nervous system mycoses. Neurology India 55(3): 191-197.

Asmundsdottir LR, Erlendsdottir H, Haraldsson G, Gus H, Xu J, Gottfredsson M. 2008. Molecular epidemiology of candidemia: evidence of clusters of smoldering nosocomial infections. Clin Infect Dis 47: e17-24.

Baddley JW, Winthrop KL, Patkar NM, Delzell E, Beukelman T, Xie F, Chen L, Curtis JR. 2011. Geographic distribution of endemic fungal infections among older persons, United States. Emerging Infectious Diseases 17(9): 1664-1669.

Baldauf SL, Roger AJ, Wenk-Siefert I, Doolittle WF. 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290: 972-977.

Ball CA and Cherry JM. 2001. Genome comparisons highlight similarity and diversity within the eukaryotic kingdoms. Current Opinion in Chemical Biology 5(1): 86-89.

Baumgardner DJ. 2009. Commentary: Environmental determinants of dimorphic systemic mycoses – the macro and the micro. International Journal of Epidemiology 38: 1649-1650.

Baxter M, Illston GM. 1980. Temperature relationships of fungi isolated at low temperatures from soils and other substrates. Mycopathologia 72(1): 21-25.

Bergman A, Casadevall A. 2010. Mammalian endothermy optimally restricts fungi and metabolic costs. mBio 1(5): e00212-10.

Blakely G, Hekman J, Chakraburity K, Williamson PR. 2001. Evolutionary Divergence of an Elongation Factor 3 from Cryptococcus neoformans. Journal of Bacteriology 183(7): 2241-2248.

Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU, Ryan RM. 2008. Vertical and horizontal transmission of *Candida albicans* in very low birth weight infants using DNA fingerprinting techniques. Pediatr Infect Dis J 27: 231-235.

Bin C, Hui W, Peng W, Meng–Tao L, Yuan–Jue Z. 2006. Clinical parameters and outcomes of *Pneumocystis jirovecii* pneumonia in non–HIV, AIDS patients. Clin Med J 119: 234–237.

Bio-Rad. 2013. Quick Start ™ Bradford Protein Assay. Bio-Rad Laboratories, Inc. Accessed online 10 Apr 2015. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf.

Brown J, Benedict K, Park BJ, Thompson III GR. 2013. Coccidioidomycosis: epidemiology. Clinical Epidemiology 5: 185-197.

Burge H. 2006. How does heat affect fungi? The Environmental Reporter 4(3). Accessed online 12 April 2015. https://www.emlab.com/s/sampling/env-report-03-2006.html.

Chang M, Jaehning JA. 1997. A multiplicity of mediators: alternative forms of transcription complexes communicate with transcriptional regulators. Nucleic Acids Research 25(24): 4861-4865.

Chayakulkeeree M, Perfect JR. 2006. Cryptococcosis. Infect Dis Clin N Am 20: 507-544.

Coelho L, Veloso VG, Grinsztejn B, Luz PM. 2014. Trends in overall opportunistic illnesses, *Pneumocystis carinii* pneumonia, cerebral toxoplasmosis and *Mycobacterium avium* complex incidence rates over the 30 years of the HIV epidemic: a systematic review. The Brazilian Journal of Infectious Diseases 18(2); 196-210.

Collins F. 2013. Yes It's True: There's Fungus Among Us. National Institutes of Health. Online. http://directorsblog.nih.gov/2013/05/28/yes-its-true-theres-fungus-among-us/. Accessed 27 December, 2014.

Colmer S. 2013. Evolutionary Characterization of *Schizosaccharomyces pombe* Eukaryotic Translation Elongation Factor 3 [Undergraduate Honors Thesis]. Madison, NJ: Drew University. 58p.

Colthurst DR, Schauder BS, Hayes MV, Tuite MF. 1992. Elongation factor 3 (EF-3) from *Candida albicans* shows both structural and functional similarity to EF-3 from *Saccharomyces cerevisiae*. Molecular Microbiology 6(8): 1025-1033.

Cox MM, Doudna JA, O'Donnell M. 2010. Molecular Biology: Principles and Practice. W.H. Freeman and Co, NY, pp. 638-647.

Dasmahapatra B, Chakraburtty K. 1981. Protein Synthesis in Yeast. The Journal of Biological Chemistry 256(19): 9990-10004.

De Boer MG, Bruijnestein Coppenraet LE, Gaasbeek A, Berger SP, Gelinck LB, van Houwelingen HC, van de Broek P, Kuijper EJ, Kroon FP, Vandenbroucke JP. 2007. An outbreak of *Pneumocystis jirovecii* pneumonia with 1 predominant genotype among renal transplant recipients: interhuman transmission or a common environmental source? Clin Infect Dis 44: 1143–1149.

De Castro N, Neuville S, Sarfati C, Ribaud P, Derouin F, Gluckman E, Socié G, Molina JM. 2005. Occurrence of *Pneumocystis jirovecii* pneumonia after allogeneic stem cell transplantation: a 6–year retrospective study. Bone Marrow Transplant 36: 879–883.

Deepe GS Jr. 1997. Prospects for the Development of Fungal Vaccines. Clinical Microbiology Reviews 10(4): 585-596.

Dever TE and Green R. 2012. The Elongation, Termination, and Recycling Phases of Translation in Eukaryotes. Cold Spring Harbor Perspectives in Biology 4: a013706.

Doolittle WF. 1999. Phylogenetic classification and the universal tree. Science 284: 2124-2128.

Elzanowski A, Ostell J. 2013. The Genetic Codes: 12 – The Alternative Yeast Nuclear Code (transl_table=12). NCBI. Accessed online 15 Mar 2015. http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi#SG12.

Enoch DA, Ludlam HA, and Brown NM. 2006. Invasive fungal infections: a review of epidemiology and management options. Journal of Medical Microbiology 55: 809-818.

Fancher LJ. 2004. Prokaryotic and Eukaryotic Cells. College of DuPage. Accessed online 12 April 2015. http://www.cod.edu/people/faculty/fancher/prokeuk.htm.

Geitz RD, Woods RA. 2002. Transformation of yeast by lithium acetate/single-stranded

carrier DNA/polyethylene glycol method. Methods Enzymology 350: 87-96.

Gouba N, Drancourt M. 2015. Digestive tract mycobiota: A source of infection. Medicin et maladies infectieuses. Pii: S0399-077X(15)00014-1 [Epub ahead of print].

Gottlieb D. 1963. Carbohydrate catabolism by fungi. Pure Applied Chemistry 7(4): 603-610.

Gray MW. 2012. Mitochondrial Evolution. Cold Spring Harbor Perspectives in Biology 4: a011403

Groll AH, Walsh TJ. 2002. Antifungal chemotherapy: advances and perspectives. Swiss Med Wkly 132: 303-311.

Guarro J, Gene J, Stchigel AM. 1999. Developments in fungal taxonomy. Clinical Microbiology Reviews 12(3): 454-500.

Gutierrez S, Respaldiza N, Campano E, Martinez-Risquez MT, Calderon EJ, De La Horra C. 2011. *Pneumocystis jirovecii* colonization in chronic pulmonary disease. Parasite 18: 121-126.

Hampsey M. 1998. Molecular Genetics of the RNA Polymerase II General Transcriptional Machinery. Microbiology and Molecular Biology Reviews 62(2): 465-503.

Havlickova B, Czaika VA, Friedrich M. 2008. Epidemiological trends in skin mycoses worldwide. Mycoses 51(Suppl 4): 2-15.

Hill J, Ian KA, Donald G, Griffiths DE. 1991. DMSO-enhanced whole cell yeast transformation. Nucleic Acids Research 19(20): 5791.

Hocker B, Wendt C, Nahimana A, Tonshoff B, Hausen PM. 2005. Molecular evidence of *Pneumocystis* transmission in pediatric transplant unit. Emerg Infect Dis 11: 330–332.

Huang L. 2011. Clinical and translational research in *Pneumocystis* and *Pneumocystis pneumonia*. Parasite 18: 3-11.

Jarvis WR. 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin Infect Dis 20: 1526-1530.

Jarvis WR, Martone WJ. 1992. Predominant pathogens in hospital infections. J Antimicrob Chemother 29(Supplemental A): 19-24.

Jenuwein T, Allis CD. 2001. Translating the Histone Code. Science 293: 1074-1080.

Kawai S, Hashimoto W, Murata K. 2010. Transformation of *Saccharomyces cerevisiae* and other fungi. Bioengineered Bugs 1(6): 395-403.

Kobayashi GS. 1996. Disease mechanisms of fungi. Medical Microbiology, 4th ed. Baron S, editor. Galveston, TX: University of Texas Medical Branch at Galveston. Chapter 74.

Koonin EV. 2014. Carl Woese's vision of cellular evolution and the domains of life. RNA Biology 11(3): 197-204.

Krishnamurthy S, Hampsey M. 2009. Eukaryotic transcription initiation. Current Biology 19(4): R153-6.

Kubitschek-Barreira PH, Curty N, Neves GWP, Gil C, Lopes-Bezerra LM. 2013. Differential proteomic analysis of *Aspergillus fumigatus* morphotypes reveals putative drug targets. Journal of Proteomics 78: 522-534.

Kumar R, Gururaj AE, Vadlamudi RK, Rayala SK. 2005. The clinical relevance of steroid hormone receptor corepressors. Clin Cancer Res 11(8): 2822-2831.

Life Technologies. 2015. 5-Fluoroorotic Acid. Thermo Fisher Scientific. Online. http://www.lifetechnologies.com/order/catalog/product/R0811. Accessed 26 Mar 2015.

Liu M, Gelli A. 2008. Elongation Factor 3, EF3, Associates with the Calcium Channel Cch1 and Targets Cch1 to the Plasma Membrane in *Cryptococcus neoformans*. Eukaryotic Cell 7(7): 1118-1126.

Maheshwari R, Bharadwaj G, Bhat MK. 2000. Thermophilic Fungi: Their Physiology and Enzymes. Microbiology and Molecular Biology Reviews 64(3): 461-488.

Manoloff ES, Francioli P, Taffe P, van Melle G, Bille J, Hausen PM. 2003. Risk for *Pneumocystis carinii* transmission among patients with pneumonia: a molecular epidemiology study. Emerg Infect Dis 9: 132–134.

Marco F, Lockhart SR, Pfaller MA, Pujol C, Rangel-Frausto MS, Wiblin T, Blumberg HM, Edwards JE, Jarvis W, Saiman L, Patterson JE, Rinaldi MG, Wenzel RP, Soll DR. 1999. Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. J Clin Microbiol 37: 2817-2828.

Marcon MJ, Powell DA. 1992. Human infections due to *Malassezia* spp. Clinical

Microbiology Reviews 5(2): 101-119.

Marr KA. 2004. Combination Antifungal Therapy: Where are we now, and where are we going? Oncology. Online. http://www.cancernetwork.com/oncology-journal/combination-antifungal-therapy-where-are-we-now-and-where-are-we-going. Accessed 26 Mar 2015.

Martin GS, Mannino DM, Eaton S, Moss M. 2003. The Epidemiology of Sepsis in the United States from 1979 through 2000 [Review]. New England Journal of Medicine 348: 1546-1554.

McNeil MM, Nash SL, Hajjeh RA, et al. 2001. Trends in Mortality Due to Invasive Mycotic Diseases in the United States, 1980-1997 [Review]. Clinical Infectious Diseases 33: 641-647.

Mean M, Marchetti O, and Calandra Thierry. 2008. Bench-to-bedside review: Candida infections in the intensive care unit [Review]. Critical Care 12(1): 204-212.

Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, Huang L, Beard CB, Kaplan JE. 2004. Current epidemiology of *Pneumocystis* pneumonia. Emerg Infect Dis 10: 1713–1720.

Nakamura Y. 2007. Codon Usage Database: *Chlamydomonas reinhardtii* [gbpln]: 846 CDS's (420455 codons). NCBI-GenBank. Accessed online 15 Mar 2015. http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.

National Toxicology Program. 2011. Selenium Sulfide CAS No. 7446-34-6. Reports on Carcinogens, 13th ed, 12: 376-377.

NCBI. 2015. Taxonomy Browser. NLM NIH. Online. http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi. Accessed 24 Mar 2015.

Nealson KH and Conrad PG. 1999. Life: Past, Present, and Future. Philosophical Transactions of the Royal Society of London 354: 1923-1939.

New England BioLabs. 2015a. Alkaline Phosphatase, Calf Intestinal (CIP). NEB. Online. https://www.neb.com/products/m0290-alkaline-phosphatase-calf-intestinal-cip#tabselect0. Accessed 26 Mar 2015.

New England BioLabs. 2015b. *Bam*HI. NEB. Online. https://www.neb.com/products/r0136-bamhi. Accessed 23 Feb 2015.

New England BioLabs. 2015c. Quick Ligation Protocol (M2200). NEB. Online. https://www.neb.com/protocols/1/01/01/quick-ligation-protocol. Accessed 26 Mar

2015.

New England BioLabs. 2015d. *Sac*I. NEB. Online. https://www.neb.com/products/r0156-saci. Accessed 23 Feb 2015.

New England BioLabs. 2015e. Phusion® High-Fidelity PCR Kit. NEB. Online. https://www.neb.com/products/e0553-phusion-high-fidelity-pcr-kit.. Accessed 26 Mar 2015.

New England BioLabs. 2015f. *Xho*I. NEB. Online. https://www.neb.com/products/r0146-xhoi. Accessed 23 Feb 2015.

Pasricha S, Payne M, Canovas D, Pase L, Ngaosuwankul N, Beard S, Oshlack A, Smyth GK, Chaiyaroj SC, Boyce KJ, Andrianopoulos A. 2013. Cell-type-specific Transcriptional Profiles of the Dimorphic Pathogen *Penicilium marneffei* Reflect Distinct Reproductive, Morphological, and Environmental Demands. Genes Genomes Genetics 3: 1997-2014.

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, Hellen CUT. 2001. Molecular mechanisms of translation initiation in eukaryotes. Proceedings of the National Academy of Sciences 98(13): 7029-7036.

Pfaller MA, Diekema DJ. 2010. Epidemiology of Invasive Mycoses in North America. Critical Reviews in Microbiology 36(1); 1-53.

QIAGEN. 2013. QIAquick PCR Purification Kit. QIAGEN. Online. http://www.qiagen.com/products/catalog/sample-technologies/dna-sample-technologies/dna-cleanup/qiaquick-pcr-purification-kit#productdetails. Accessed 9 Oct 2014.

Qin S, Xie A, Bonato CM, McLaughlin CS. 1990. Sequence analysis of the translational elongation factor 3 from *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 265(4): 1903-1912.

Rabodonirina M, Vanhems P, Couray–Targe S, Gillibert RP, Ganne C, Nizard N, Colin C, Fabry J, Touraine JL, van Melle G, Nahimana A, Francioli P, Hauser PM. 2004. Molecular evidence of interhuman transmission of *Pneumocystis* pneumonia among renal transplant recipients hospitalized with HIV–infected patients. Emerg Infect Dis 10: 1766–1773.

Rees JR, Pinner RW, Hajjeh RA, Brandt ME, Reingold AL. 1998. The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992-1993: results of a population-based laboratory active surveillance. Clin Infect Disease 27: 1138-1147.

Ro BI, Dawson TL. 2005. The role of sebaceous gland activity and scalp microfloral metabolism in the etiology of seborrheic dermatitis and dandruff. The Journal of Investigative Dermatology Symposium Proceedings 10(3): 194-197.

Roblot F, Imbert S, Godet C, Kauffmann C, Ragot S, Le Moal G, Roblot P, Rodier MH, Robert R, Becq–Giraudon B, Guilhot F. 2004. Risk factors analysis for *Pneumocystis jirovecii* pneumonia (PCP) in patients with haematological malignancies and pneumonia. Scand J Infect Dis 36: 848–854.

Roth RR and James WD. 1988. Microbial ecology of the skin. Annual Reviews in Microbiology 42: 441-464.

Roy D, Paul A, Roy A, Ghosh R, Ganguly P, Chaudhuri S. 2014. Differential acetylation of histone H3 at the regulatory region of *OsDREB1b* promoter facilitates chromatin remodeling and transcription activation during cold stress. PLOS One 9(6): e100343.

Sarthy AV, McGonigal T, Capobianco JO, Schmidt M, Green SR, Moehle CM, Goldman RC. 1998. Identification and kinetic analysis of a functional homolog of Elongation Factor 3, YEF3 in *Saccharomyces cerevisiae*. Yeast 14: 239-253.

Sasikumar AN, Kinzy TG. 2014. Mutations in the Chromodomain-like Insertion of Translation Elongation Factor 3 Compromise Protein Synthesis through Reduced ATPase Activity. The Journal of Biological Chemistry 289(8): 4853-4860.

Sigma-Aldrich. Magnesium chloride solution – Product Information. Sigma-Aldrich. Saint Louis, Missouri. Online. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/2/m8787dat.pdf. Accessed 17 Sept 2014.

Sonenberg N, Hinnebusch AG. 2009. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Tests. Cell 136(4): 731-745.

Tisch D, Schmoll M. 2010. Light regulation of metabolic pathways in fungi. Applied Microbiology and Biotechnology 85: 1259-1277.

UniProt. 2014. Alignment [completed] - EF3 of Sc vs. Cr. http://www.uniprot.org/align/2014091893GHN00740. Accessed 18 Sept 2014.

Vannini A, Cramer P. 2012. Conservation between the RNA Polymerase I, II, and III Transcription Initiation Machineries. Molecular Cell 45: 439-446.

Vetter J. 1997. Toxins of *Amanita phalloides*. Toxicon 36(1): 13-24.