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Expression in Yeast of the Metacaspase Scp3 from the Fungus Schizophyllum commune

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Expression in Yeast of the Metacaspase Scp3 from the Fungus *Schizophyllum commune*

by

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ABSTRACT

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ADVISOR: Kristin M. Fox

Metacaspases are caspase homologs that are found in plants, fungi, and protists. They are involved in programmed cell death, a structured and regulated process to break down cells. There is limited information available on metacaspases and further research is necessary to understand how they work. One reason metacaspases are studied is that they are considered to be a potential drug target for pathogenic microorganisms. Five metacaspases scp1-5 were identified in *S. commune* through their similarity to the yeast metacaspase Yca1. The overall goal of the lab is to study and characterize these proteins. This thesis outlines the path taken to express the *Schizophyllum commune* metacaspase scp3 in yeast. Previous efforts in our lab to express metacaspases in *E. coli* have not been successful. I hypothesized that yeast would be a more desirable expression vehicle due to its closer relationship to *S. commune*; a more similar environment could yield more active protein. Scp3 has been successfully cloned into a yeast expression vector pYES2.1/V5-His-TOPO® and β-galactosidase expression has been obtained in yeast with the expression control vector pYES1.2/V5-His/*lacZ*. Future work on this project will be to perform a point mutation in the expression plasmid to remove a stop codon before the scp3 sequence.

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INTRODUCTION

Programmed Cell Death and Apoptosis

Programmed cell death (PCD) is a controlled process in which a cell dies by breaking itself down. It is distinguished from other types of cell death by the fact that it is highly regulated and structured. The most common form of PCD is apoptosis, a pathway that is initiated and coordinated by proteins known as caspases.[1,2] When apoptosis is induced, the chromosomes start to condense, the nuclear envelope disintegrates, DNA fragments, and the plasma membrane bulges out. Eventually, the cell breaks into multiple membrane-enclosed sacs that are then engulfed by other cells.

Apoptosis occurs in many situations, including body patterning during development and during a response to stress. In addition, it is essential for the removal of damaged, aged, or superfluous cells.[3] Stressors that induce apoptosis include osmotic pressure, oxidative stress, toxins, viruses, and aging.[4] Apoptosis is initiated through either the extrinsic or the intrinsic pathway. The extrinsic pathway is initiated by outside signals from a neighboring cell that selected the other cell to undergo apoptosis. The intrinsic pathway occurs when the cell ceases to receive the constant external signals that normally suppress apoptosis.[1] Problems with the apoptotic process may lead to diseases such as cancer and neurodegenerative disorders.[5]

Apoptosis is a conserved construct, having evolved from a process in ancient eukaryotic organisms. However, there are multiple hypotheses as to how PCD evolved starting in single-celled organisms. One theory is that it was a way for a less healthy cell to sacrifice itself to benefit the colony, such as when there was a limited nutrient supply. On the other hand, there is no advantage for a single cell to undergo PCD; early death of an organism would not be selected for. It is therefore concluded that there must be other, non-PCD roles of the proteins that evolved

in the PCD pathway.[2] As a result, caspases and metacaspases, the proteins responsible for PCD, hold other important roles in addition to programmed cell death, even in multicellular organisms.

Caspases

Caspases are proteins classified as proteases, meaning they cleave other proteins. Caspases are significant because they are responsible for apoptosis in metazoans. They are also involved in many processes other than cell death, including inflammation, cell cycle control, cell proliferation and cell differentiation.[4,6] Due to their multifunctionality, caspases have many targets consisting of transcription and elongation factors, ribosomal proteins, and structural molecules.[4] In fact, a single caspase may have hundreds of different targets that it is able to discriminate.[7]

Directly after translation, caspases exist as inactive zymogens that must be cleaved in between the p20 and p10 subunits in order to be activated.[8,9] p20 is a large catalytic domain on the N-terminal side of the protein, and p10 is a small domain on the C-terminus.[9] The cleavage is either carried out autocatalytically by the caspase itself or performed by another caspase.[8] The final structure is a catalytic dimer consisting of two of each subunit with two identical active sites.[7] The active sites consist of a cysteine-histidine catalytic diad[1,3,9] Once active, caspases cleave polypeptides, with specificity for cleaving after aspartic acid. This specificity is mainly a result of the S1 specificity pocket that contains two arginines and a glutamine, creating a spot of positive charge that complements the negatively charged aspartic acid.[10]

While caspases contain the same basic structures, they are grouped into effector or initiator caspases depending on their sequences and the order in which they are activated in PCD.[8] Initiator caspases contain a large prodomain, a sequence on their N-terminus that codes for constituents such as the caspase recruitment domain (CARD) and the death effector domain

(DED) that are involved in protein-protein interactions responsible for guiding caspases into different cell death signaling pathways.[8] However, prodomains are not necessarily required for caspase activity.[5] The role of initiator caspases is to start the extrinsic and intrinsic pathways toward apoptosis.[7] In addition, initiator caspases activate the effector caspases, which do not contain a significant prodomain, through cleavage.[5,9] Both of the pathways converge with the activation of the effector caspases that perform most of the cleavages that result in the physical characteristics of apoptosis.[7]

Due to the fact that apoptosis, and therefore caspases, are involved in a variety of diseases, caspases are promising drug targets. Already it has been shown that caspase inhibitors are able to improve stroke and brain injury symptoms in animals.[5]

Metacaspases

Metacaspases are caspase homologs that are found in plants, fungi, and protozoa; they are responsible for initiating PCD in these organisms.[9,11] Two classes of caspase-like proteins, metacaspases and paracaspases, were first identified in 2000 by Uren et al. by the similarity of their sequences to caspases. Paracaspases, like caspases, are found in animals. On the other hand, metacaspases, the focus of our research, are found in plants, fungi, and protozoa, organisms that do not contain caspases. These three proteins share a common ancestor from early in the evolution of eukaryotes and are derivatives of ancient cysteine proteases.[8]

Metacaspases are sequence homologs to caspases and therefore have some structural similarities, such as the p20 and p10 subunits.[12] Metacaspases are also split into two subsets, type I and type II. Type I metacaspases contain a prodomain that is similar to the CARD and DED domains of initiator caspases, whereas type II metacaspases do not contain a prodomain, like the

effector caspases.[8] However, the distance between the p20 and p10 subunits in the Type II

metacaspase is longer than that of the effector caspase by about 130 amino acids.[9]

Recently, another form of metacaspases was discovered in phytoplankton, Type III,

where the p10 subunit lies on the N-terminus instead of the C-terminus. In addition to the Type

III metacaspases, metacaspase-like proteases and Type I metacaspases lacking a prodomain were

also found. This hints that the classification of metacaspases is more uncertain than previously

thought.[9] Depictions of the three types of metacaspases are shown in Figure 1.

Figure 1. Diagram of the domains in type I, II, and III metacaspases. Type I metacaspases
contain a prodomain on the N-terminus and Type III has p10 and p20 subunits in reverse order.
Scp3, a Type II metacaspase, is indicated.

In addition to the subunits and prodomains, the cysteine-histidine active site of caspases

is also conserved in metacaspases. However, the specificity of metacaspases is different:

metacaspases cleave after lysine and arginine instead of aspartic acid.[12] This is a significant

difference because lysine and arginine are basic amino acids. The substrate specificity is

determined by a binding site structure similar to the S1 pocket of caspases, indicating that the

recognition mechanism for metacaspases and caspases is related.[13]

While the activation mechanism of metacaspases is unknown, it is likely different than

that of caspases. For instance, autocatalytic processing has been observed in metacaspases, but it

is not necessarily essential for activity. In fact, the crystal structures of some type I metacaspases show that they are monomers, not dimers like active caspases.[9] The core hemoglobinase fold in metacaspases contains a β-sheet made up of eight strands, instead of the six in caspases. This structural difference prevents the dimerization of metacaspases. It also appears that metacaspases are regulated after translation in multiple ways, sometimes by other metacaspases.

Furthermore, metacaspase activity is highly dependent on cell conditions.[4] For instance, the activity of some metacaspases is dependent on calcium, although excessive concentrations may cause a decrease in activity.[14,15] In addition, higher temperatures increase autoprocessing, which, if uncontrolled, will ultimately lead to inactivation. Metacaspases also observe a pH dependency similar to that of caspases, with the enzyme most active when the catalytic cysteine is deprotonated.[15]

Metacaspases have been proven to be necessary for the induction of certain forms of PCD in various fungi, protozoa, and plants. However, the pathway of cell death and the exact role of metacaspases are still unclear.[16] While metacaspases appear to be analogous to caspases in many ways, it is unknown if metacaspases act exactly as caspases in the apoptotic pathway.[9] Since metacaspases are a relatively recent discovery, they are currently being studied to learn more about their structures, how they work, and the processes in which they are involved. The intention is that metacaspases will be able to be used as chemotherapy agents and as other drug targets. Metacaspases are medically relevant due to their involvement in cell death, as well as the fact that they are significantly different than caspases and are not found in humans.[14]

Metacaspases and Non Cell Death Activity

It has already been established that caspases are involved in cell cycle progression, replication, cell movement, and cell differentiation in addition to apoptosis.[6] The further

metacaspases are studied, the more information that is revealed indicating their multiple roles in plants, fungi and protozoa. Like caspases, metacaspases are known to be involved in cell cycle progression.[11] Disrupting metacaspase expression, either by overexpression or deletion, results in stunted growth in *Leishmania major*. The cell cycle in yeast was also shown to be slowed with a deletion of its metacaspase Yca1.[16] In addition, Yca1 is involved in proteostasis by breaking down proteins. Proteostasis is the balance of protein production with degradation and ensures that misfolded proteins are properly managed. Defects in this process result in insoluble protein aggregates, which are associated with Alzheimer's, Huntington's, and Parkinson's Disease. Yca1 is also possibly involved in ribosome biogenesis and protein synthesis.[6] The role of metacaspases in apoptosis has already implicated them as a drug targets; the additional roles of metacaspases that are being discovered further increases the possibility of their usefulness in medicine.

Schizophyllum commune

The metacaspases being studied by the Fox Lab come from the model organism *Schizophyllum commune,* a split gill mushroom that is used to study fungal genetics.[17] *S. commune* genetics are studied for various reasons, including being the only mushroom-forming fungus that has had genes inactivated by homologous recombination. In addition, its recombinant DNA may be expressed in other fungi. *S. commune* is also relatively unique in that it is a mushroom that may be grown on artificial media. It also has a reasonably short life cycle of 10 days and may be found on every continent except Antarctica. Professor Kristin Fox identified five metacaspases from *S. commune*, Scp1, Scp2, Scp3, Scp4, and Scp5. These proteins were determined to be metacaspases by a sequence alignment with yca1, the metacaspase from *S. cerevisiae*.[18] The sequence alignment of Scp3 with Yca1 is shown in Figure 2.

```
yca1    MYPGSGRYTYNNAGGNNGYQRPMAPPPNQQYGQQYGQQYEQQYGQQYGQQNDQQFSQQYA 60
scp3    ------------------------------------------------------------

yca1    PPPGPPPMAYNRPVYPPPQFQQEQAKAQLSNGYNNPNVNASNMYGPPQNMSLPPPQTQTI 120
scp3    ------------------------------------------------------------

yca1    QGTDQPYQYSQCTGRRKALIIGINYIGSKNQLRGCINDAHNIFNFLTNGYGYSSDDIVIL 180
scp3    -------MESHCTGRKRAICIGIDYQGQSSELKGCINDARDVRDFLVKRCRFRKEDVMLL 53
                *:****::*: ***:* *...:*:******::: :**.:   : .:*:::*

yca1    TDDQNDLVR-VPTRANMIRAMQWLVKDAQPNDSLFLHYSGHGGQTEDLDGDEEDGMDDVI 239
scp3    TDDKSRSSRSYPSRANMISAMKWLVEGAQMHDSLFLHYSGHGGQKRDFNDDEADGYDETI 113
        ***:.     *  *:***** **:***:.** :*************..*::.** ** *:.*

yca1    YPVDFETQGPIIDDEMHDIMVKPLQQGVRLTALFDSCHSGTVLDLPYTYSTKGIIKEPNI 299
scp3    FPVDHEEAGVIIDDEMHKILVDPLPAACRLTALFDSCHSGTVLDLPYIYSQHGRLR---- 169
        :***.*   * *******.*:*.**   . ****************** ** :* ::

yca1    WKDVGQDGLQAAISYATGNRAALIGSLGSIFKTVKGGMGNNVDRERVRQIKFSAADVVML 359
scp3    ------------------------------------GSHVS-PRARLRQASPADVITW 190
                                            *.:*.  *.*  : * *.***:

yca1    SGSKDNQTSADAVEDGQNTGAMSHAFIKVMTLQPQQSYLSLLQNMRKELAGKYSQKPQLS 419
scp3    SACRDGQTSVDTFAGGEAVGAMSYAFLKILRNERDLSYSDLLKRLR-------------- 236
        *..:*.***.*:. .*: .****:**:*::  : : ** .**:.:*

yca1    SSHPIDVNLQFIM 432
scp3    -------------
```

Figure 2. ClustalW2 alignment of the amino acid sequence for Yca1 and Scp3. The purple box designates the histidine and cysteine residues that form the active site. The asterisks indicate where the amino acids are the same, two dots show where they are very similar, one dot is somewhat similar.

The percent identity for Yca1 and Scp3 is 52%, meaning that they are homologous. The most significant similarity, shown with Scp3 in Figure 2, is that the *S. commune* metacaspases contain the conserved cysteine-histidine catalytic diad. One general difference between the two metacaspases is that Yca1 is almost 200 amino acids longer than scp3. In addition, Yca1 is a type I metacaspase, making it more similar to scp1, 2, 4, and 5 than scp3, which is Type II and has a truncated prodomain. Yca1 differs from the *S. commune* metacaspases in that it is the only metacaspase in *S. cerevisiae*. In addition, yeast is single-celled, whereas *S. commune* is multicellular.[19] By studying the metacaspases in *S. commune*, we can determine if other fungal metacaspases share similar characteristics and roles with those of yeast.

Yeast as a Microorganism for Studying Proteins

The metacaspases we are studying come from *S. commune*, but we cannot access them directly because they exist in too small of a concentration to be easily isolated and studied. Therefore, we use microorganisms to produce the proteins of interest, typically resulting in much higher protein concentrations.

The yeast *Saccharomyces cerevisiae* is a microorganism commonly used for the expression of foreign proteins.[20] *S. cerevisiae* contains a metacaspase of its own, yca1, but for this project, it is being used for its role as a classic model organism of eukaryotic systems.[21] While only single-celled, many characteristics of yeast remain conserved in higher eukaryotic organisms.[20] While yeast is genetically more complex than bacteria, it still possesses some of the same attributes that make bacteria so useful and popular in biological research. Yeast is single-celled, may be grown quickly in artificial media, and has a genome that is both small and entirely sequenced.[20, 21,22]

My Project

The long-term goal of metacaspase research is to harness the roles of metacaspases in cell death and other fundamental processes for use in medicine. Currently, work persists to further characterize metacaspases, especially in reference to caspases. In addition, there is a lot left to learn about how metacaspases function and their part in the PCD pathway.

The overall goal of the Fox Lab is to express and characterize five metacaspases scp1, scp2, scp3, scp4, and scp5 from the fungus *Schizophyllum commune*. The most recent work has focused on the expression of scp3 in a soluble and active form.

The metacaspase I am studying for my research is Scp3, which is type II and therefore has a very short prodomain. Previously, only Type I metacaspases have been found in fungi.[16] The short prodomain has resulted in more successful Scp3 expression; the other scp constructs we have worked with, 1, 2, and 5, could not be expressed without the prodomain deleted. The scp3 gene was successfully cloned into the vector pQE-80 and expressed in *E. coli* using IPTG by Dillon Bentancourt.[23] Connor Gagliardi was also able to purify scp3 with a Ni^{2+} column, but he was unable to obtain significant evidence of proteolytic activity.[18] The focus of Ethan Loew's '13 thesis was to express the p10 and p20 subunits independently, unfold them and refold them together into their final construct. The p20 subunit was successfully expressed, but work continues to express the p10 subunit.[24] One part of Connor Gagliardi's research was the optimization of the expression of scp3 in *E. coli*, using both IPTG overexpression and autoinduction methods.[18] The use of IPTG overexpression has been successful in producing large amounts of protein, but it existed in insoluble aggregates. Through autoinduction, more soluble protein has resulted, but in amounts too small to be useful for analysis.

The aim of my project is to use *S. cerevisiae* instead of *E. coli* as a vehicle for scp3 production. The reasoning is that *S. cerevisiae*, a fungus, is physiologically more closely related to *S. commune* than is *E. coli*. Therefore, *S. cerevisiae* may yield a more appropriate environment for the metacaspases, allowing them to be expressed in a soluble and active form. In addition,Machado et al. have shown that the stability of a *Trypanosoma brucei* metacaspase decreased more at 37˚C, the growth temperature of *E. coli*, than at 30˚C, the growth temperature of yeast.[15] Another possibility is that it may be possible to express the other metacaspases with their full prodomain, something that was not possible through *E. coli* expression. Being able to work with the full metacaspases would allow us to learn more about how the *S. commune*

metacaspases are structured and activated.

Techniques

PCR

Polymerase chain reaction (PCR) is a method used to amplify a small segment of DNA. Double-stranded DNA that contains the target sequence is used as a template. First, the DNA is heated to 95˚C to separate the two strands. In the next step, the temperature is reduced to 50-60˚C to allow for the primers to anneal to the DNA on either side of the target sequence. Primers are short pieces of single-stranded DNA that are designed to complement the target sequence and to direct the DNA polymerase so it only copies the desired sequence. Once the primers are annealed, the polymerase synthesizes two new strands that are complementary to the template DNA. As these three steps are repeated, the amount of the gene of interest increases exponentially. After 20 cycles, there will be more than a million copies of the target sequence.[1]

Plasmids

A plasmid is a small circular piece of DNA that may be fabricated in a laboratory and inserted into bacterial or yeast cells. These plasmids are often small and easily replicated. Plasmids frequently contain traits such as antibiotic resistance to allow the identification of the cells that contain the plasmid. Antibiotic resistance is an example of a selectable marker.[1] The plasmid used for my research is pYES2.1/V5-His-TOPO from the pYES2.1 TOPO® TA Yeast Expression Kit (Invitrogen). A diagram of the plasmid may be seen in Figure 3.

512...513 scp3
543...584 V5 epitope
594...611 6x His

pYES2.1/V5-His-TOPO

1886...2746 Ampicillin

URA3 2764...3645
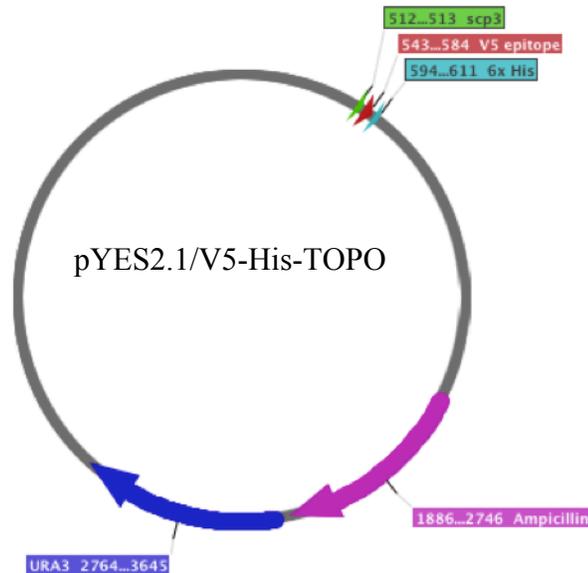
Figure 3. Diagram of pYES2.1/V5-His-TOPO. The sites of ampicillin resistance, URA3 gene, his tag, V5 epitope, and the point where scp3 is ligated in.

pYES2.1/V5-His-TOPO can be inserted into both *E. coli* and yeast. To select for plasmid-containing *E. coli,* pYES confers resistance to ampicillin, an antibiotic. In order to select for yeast that contains the plasmid, pYES contains a URA3 gene. The URA3 gene is responsible for the production of uracil, an essential building block of DNA. The strain of yeast I am working with contains a deletion for this gene; therefore, it will only be able to grow in a uracil-free medium if it contains the plasmid.

In addition to markers, plasmids often contain restriction sites that allow the plasmid to be cut, either for the insertion or the removal of a gene. Conversely, pYES2.1/V5-His-TOPO does not need to be cut first in order to insert a gene. Instead, it is linear, with an overhanging

thymine on each edge. Overhanging adenines are added to the edges of the PCR product. These adenines interact with the unpaired thymines in the plasmid, forming a complete, circular plasmid.

Other aspects of pYES2.1/V5-His-TOPO include an additional 6 histidines that add to the C terminus of the expressed protein. This tag of histidines may be used to purify the protein using a Ni^{2+} affinity column. In addition, a V5 epitope is added to the protein. The V5 epitope is recognized by a V5 antibody, which may be used as the primary antibody when performing a western blot, a method for detecting specific proteins.

Lastly, the kit also supplied an expression control plasmid, pYES2.1/V5-His/lacZ. This plasmid contains the gene for β-galactosidase, whose expression may be used to verify the methods used to transform the plasmid and express and detect the protein of interest.

Transformation

The purpose of plasmids is to introduce foreign DNA into a microorganism. This is done through the process of transformation, which is the uptake of a plasmid by a cell. Transformations are often performed either through chemical treatment or by electroporation. The first step of a transformation is weakening the cell walls to permit the plasmid to enter the cell. In chemical transformation, the cells are heat shocked to induce transformation, and for electroporation, an electric current is used. Once the plasmid is inside the cell, the microorganism will create copies of the plasmid along with its own DNA as it replicates. In addition, the cells will be able to express the genes in the plasmid. Transformation is not an extremely efficient process, but even if only a few cells take up the plasmid, they will proliferate, resulting in millions of cells that contain the plasmid. The plasmid may then be harvested by

separation from the other cell components and genomic DNA. A diagram of the transformation

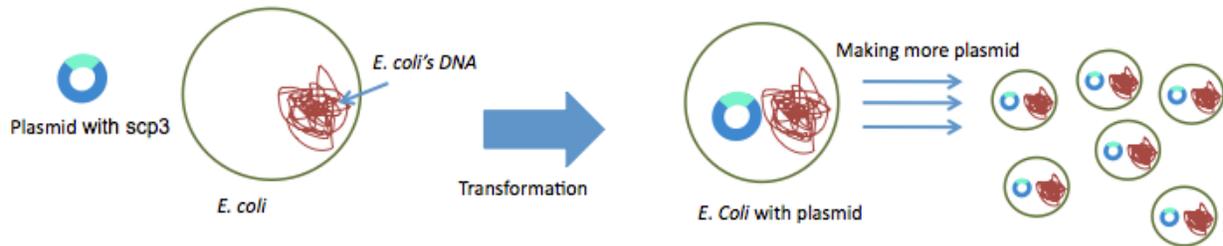process of a vector containing scp3 into *E. coli* is shown in Figure 4.

Figure 4. Diagram showing the transformation of a plasmid into *E. coli*.

Gel Electrophoresis

Gel electrophoresis is used to separate and analyze proteins and DNA fragments based on

size. Both of these methods use an electric current to pull the negatively charged molecules

through a gel matrix. Smaller molecules are faster to travel through the gel and larger molecules

are slower. Different concentrations of agarose are used depending on the size of the DNA

fragments being separated. The gel is stained with ethidium bromide, which intercalates into the

DNA and fluoresces under UV light. Each band on the gel represents a DNA fragment of a

particular size. Proteins are analyzed through sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE). The SDS denatures the proteins and surrounds them with negative

charge so that the protein separation is only by size, not shape or charge. The gel is soaked in a

stain that binds to the protein.[1]

Western Blotting

Western blotting is a method used to detect one specific protein from an SDS-PAGE that

contains many other proteins. First, the proteins from the SDS-PAGE gel are transferred to a

membrane. In order to detect the protein of interest on the membrane, western blots require the

use of antibodies. Antibodies are molecules used by the immune system to identify foreign substances and may be generated in a laboratory to target specific proteins. The western blot membrane is bathed in an antibody that only binds the protein of interest. The membrane is then bathed in a second antibody that binds the first antibody. The secondary antibody contains a chemiluminescent compound that allows for the visualization of the protein. The result is a membrane with only one band where the protein of interest is, instead of the many bands that appear on an SDS-PAGE gel.[1]

Protein Expression via GAL1 Promoter

The expression plasmid pYES2.1/V5-His-TOPO contains a GAL1 promoter in front of the target protein sequence. In the presence of glucose, the native yeast protein Gal80 binds to the GAL1 activator, Gal40, repressing protein expression. When yeast is grown in the presence of galactose, Gal80 is no longer produced and the GAL1 is free, resulting in the expression of the target protein.

The Fox Lab is studying metacaspases from *S. commune* in order to contribute to the current knowledge about the structure and function of metacaspases. Previous work to express metacaspases in *E. coli* has proven unsuccessful, so my goal was to find a solution to the expression problems faced previously. The purpose of my project was to use pYES to express Scp3 in yeast in order to obtain substantial amounts of functional protein. The techniques mentioned above were the foundation for my work to reach my goal of expressing Scp3 in yeast.

METHODS

Protocols have been adapted from edition 2.7 of the Fox Lab Protocols (August 2013), the

pYES2.1 TOPO® TA Expression Kit manual (April 2004), *Current Protocols in Molecular*

Biology, and *Current Genetics*. Modified versions of these protocols, other methods, and recipes

may be found in the Appendix.

Amplification and Isolation of scp3

Scp3 was amplified from the genomic DNA of *S. commune*. A set of primers was designed to

enable TA cloning into the pYES2.1 TOPO® vector.

Table 1. The primers used to amplify scp3, given in the 5' to 3' direction. The bolded nucleotides
are padding to ensure in frame ligation. The other nucleotides are complementary to the template
DNA.

Upstream primer	**CA**ATGGAGTCGCATTGTAC
Downstream primer	CCGTAGGCGCTTCAGCA

The Qiagen Hotstar Taq DNA Polymerase kit was used for the scp3 amplification. Using the

suggested protocol provided as a starting point, different PCR conditions including various

annealing temperatures and the presence of Q solution were tested to optimize the amplification.

The final protocol is shown in Table 2.

Table 2. PCR amplification of scp3 setup.

	µL/tube
5x PCR buffer	10
Upstream Primer	5
Downstream Primer	5
Sterile Barnstead Water	26.5
Hotstar Taq Polymerase	1
V109 cDNA 1:500 dilution	2.5

A Biorad iCycler was used with the program "hostar1" saved under the user "annekaminski"

with a hotstart at 95˚C. The samples were placed in row E to allow an annealing temperature of

55˚C. The cycles and steps are shown in Table 3.

Table 3. PCR program "hotstar1" cycles.

Cycle	Repeats	Steps	Temperature (˚C)	Time
1	0		94	3 minutes
2	35	1	94	30 seconds
		2	50	1 minute
		3	72	1 minute
3	0		72	10 minutes
4	0		18	∞

The specific ingredients in the 5x PCR buffer and the Q solution may be found in the Qiagen Hotstar Taq DNA Polymerase kit manual. Immediately following the PCR reaction, A-tailing was performed with 0.2 µL Taq per reaction (Fox Lab Protocol 2-10).

The PCR samples were electrophoresed on a 1% agarose gel and the band of spc3 was isolated from the gel. The amplified scp3 gene was then purified using the Qbiogene GENECLEAN Turbo kit (Fox Lab Protocols 3-1 and 3-2).

Ligating scp3 into pYES2.1/V5-His-TOPO®

The method of ligation was modified from the pYES2.1 TOPO® manual. 20µL of the geneclean product was combined with 1µL of the vector and 4µL salt solution provided with the kit. Other amounts of geneclean product may be used, but the salt concentration must stay at 1/6 of the ligation mixture. The reaction was incubated at room temperature for 5 minutes then set on ice and precipitated (Fox Lab Protocol 2-5). While this method was initially successful, it was unable to be reproduced.

Transformation of Vector into DH5α

The vector containing scp3 was transformed by electroporation into electrocompetent DH5α *E. coli* cells (prepared and frozen August 2012). The cells were plated on pre-warmed agar plates containing 50 µg/mL carbenicillin-Camp (Fox Lab Protocol 1-9).

Colony PCR

A colony PCR was performed to test for the presence of scp3 in the transformed DH5α cells (Fox Lab Protocol 2-8). The primers indicated in Table 1 were used with the PCR program hotstar1 (Table 3). 80 colonies were tested, divided into 8 PCR reactions.

Miniprep and Vector Analysis

Several colonies were selected for further analysis. Cells from each colony were grown overnight at 37°C in 5mL LB containing 50µg/mL CAMP. The cells were lysed and the plasmid DNA was separated from the genomic DNA of *E. coli*, using the Promega Wizard® Plus SV Minipreps DNA purification System (Fox Lab Protocol 2-1).

The vectors were then digested with restriction enzymes Pst I and Sal I. The digest recipe is listed in Table 4.

Table 4. Single tube recipe for the digest of pYES2.1/V5-His-TOPO® after scp3 ligation.

	µL/tube
Buffer D	2.0
Acetylated BSA	0.2
Sterile Barnstead Water	15.8
PstI	0.5
SalI	0.5
Miniprep DNA	1.0

The reaction was incubated at 37°C overnight then electrophoresed on a 1% agarose gel (Fox Lab Protocols 2-3 and 3-1). Minipreps that were shown by the digest to have scp3 ligated in correctly were sent to the SUNY Center for Functional Genomics for sequencing (Fox Lab Protocols 2-9).

Transformation of pYES2.1/V5-His-TOPO® Into Yeast

The yeast transformation protocol may be found in the Appendix. Both pYES containing scp3 and the expression control vector with β-galactosidase were inserted into yeast. 1.5mL of an overnight culture of *S. cerevisiae* BY4741 in 10mL YPD medium were centrifuged in an Eppendorf tube and resuspended in 100μL One Step Buffer, recipe in Table 5.

Table 5. One Step Buffer recipe.

Solution	Final concentration
Lithium Acetate	0.2 M
PEG	40%
DTT	100 mM

2μL of pYES miniprep DNA diluted 1:10, and 5.3μL of 10mg/mL salmon sperm DNA were added to the cells. This mixture was then vortexed and incubated in a 45°C water bath for 30 minutes. The cells were plated onto uracil dropout plates and incubated at 30°C for three days. The recipe for uracil dropout media is listed in Table 6.

Table 6. Recipe for uracil dropout media.

Reagent	Percent of medium	Concentration of Stock Solution
Yeast Nitrogenous Base (with ammonium sulfate, no amino acids)	0.67	N/A
Glucose	2	20%
Adenine	0.01	50x
Arginine	0.01	100x
Cysteine	0.01	N/A
Leucine	0.01	50x
Threonine	0.01	100x
Tryptophan	0.01	50x
Aspartic Acid	0.005	50x
Histidine	0.005	100x
Isoleucine	0.005	100x
Methionine	0.005	100x
Phenylalanine	0.005	100x
Proline	0.005	100x
Serine	0.005	100x
Tyrosine	0.005	N/A
Valine	0.005	100x

The medium was prepared using 100x or 50x stock solutions of the reagents except yeast

nitrogenous base, cysteine, and tyrosine, which were added as solids. The glucose was

autoclaved separately as a 20% solution. For plates, agar was added at a concentration of 2%

after the other reagents were dissolved.

Expression of β-galactosidase

Two colonies of transformed yeast were first cultured separately in 10mL of a primary culture,

20% glucose uracil dropout medium. The cultures were incubated for two days in 50mL

Erlenmeyer flasks at 30°C, with shaking at 250-300 rpm. It was necessary for the 50mL

induction culture to begin growth with an OD_{600} of 0.4. Therefore, the OD_{600} of the primary

culture was used to calculate the volume of culture to add to the induction medium. A sample

calculation is demonstrated in the Appendix. The determined amount of primary culture was

centrifuged so that the glucose medium could be removed. The cells were then resuspended in 2mL of the 20% galactose induction medium, which was then transferred to 50mL of the induction medium in 250mL Erlenmyer flask. The induction medium consisted of uracil dropout medium with 20% galactose instead of glucose. The culture was grown at 30˚C for 24 hours, with 5mL samples of culture taken at various time points ranging from 0 to 30 hours. The samples were pelleted and stored at -80˚C.

Yeast Lysis

The frozen cell pellets were resuspended in breaking buffer (Table 7).

Table 7. Yeast breaking buffer recipe.

EDTA	1 mM
Glycerol	5%
PMSF	1 mM
Sodium Phosphate, pH 7.4	50 mM

The cells were then vortexed with 0.3 g glass beads for 30 seconds, then set on ice for 30 seconds. This was repeated four times. The cells were then centrifuged and the lysate was removed.

SDS-PAGE and Western Blot

The yeast lysate was electrophoresed on a 12% acrylamide gel (Fox Lab Protocol 3-3). This gel was then used to perform a western blot (Fox Lab protocol 3-4). The primary antibody was mouse anti-V5 (Novex 1408864) and the secondary antibody was goat anti-mouse (Invitrogen 626520).

RESULTS AND DISCUSSION

Amplification of scp3

Scp3 was amplified from *S. commune* V109 cDNA using PCR. The PCR was performed with

Hotstar Taq, at annealing temperatures of 50˚C (without Q solution), 55˚C, and 60˚C (with Q

solution).[*] The PCR was analyzed using a 1% agarose gel and the results are shown in Figure 5.
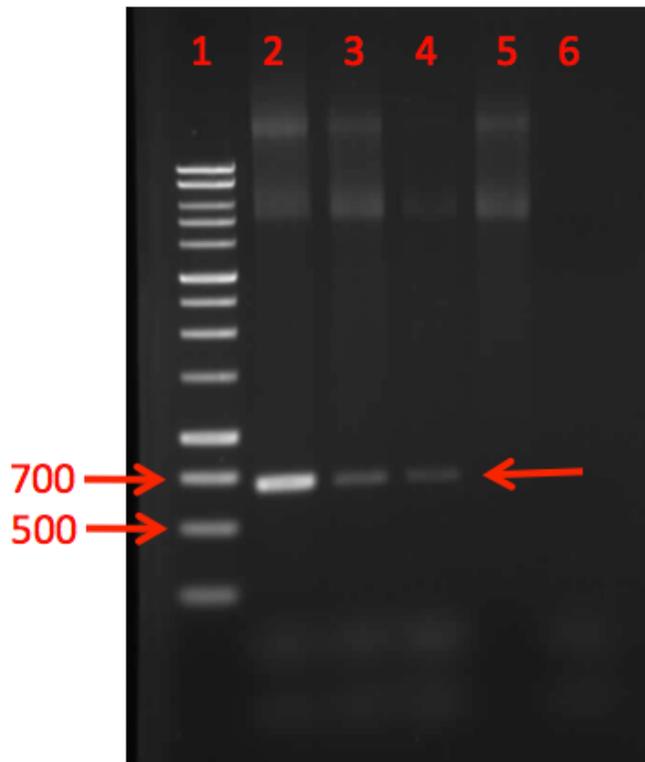

Figure 5. 1% agarose gel showing amplification of scp3. Lane 1 is the 1kb molecular weight marker. The PCR reaction in lane 2 did not contain Q solution and had an annealing temperature of 50˚C. PCR reactions in lanes 3 and 4 contained 19μL Q solution and were performed with annealing temperatures of 55 and 60˚C respectively. The control PCR with no primers is in lane 5 and the control with no template is in lane 6. Amplified scp3 is shown at 711 bp, indicated with an arrow. Lane 1 is a 1kb ladder

The bands at 711 bp are amplified scp3, denoted with the arrow. The bands at the top of the gel

in lanes 2 through 5 are the template DNA and the bands in lanes 2,3,4, and 6 are the primers.

From this gel, it was determined that the best PCR method was an annealing temperature of 50°C

with a reaction without Q solution (Figure 5, lane 2) since the scp3 band was the brightest.

Ligation of scp3 into pYES and transformation into *E. coli*

The amplified scp3 gene was successfully ligated into pYES through TA cloning. The ligation

resulted in many colonies containing scp3, as shown by the colony PCR in Figure 6.
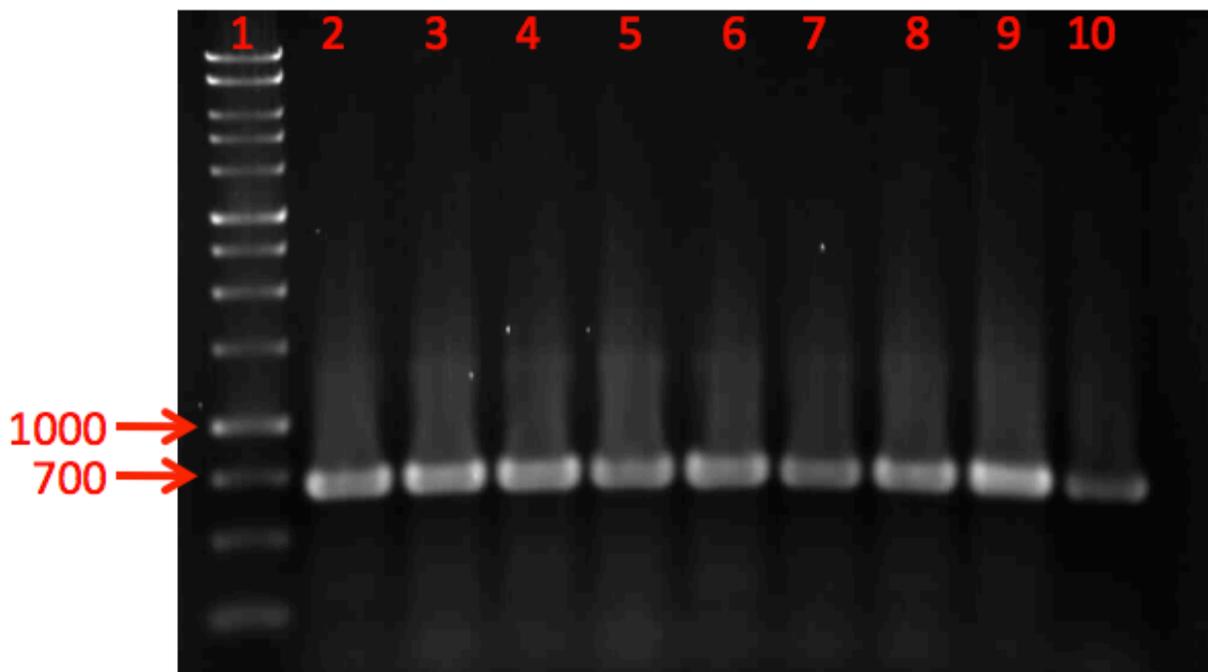
Figure 6. 1% agarose gel of the colony PCR showing scp3 present in every reaction. Lane 1

isthe 1Kb molecular weight marker and lane 10 is the blank PCR reaction with no template

DNA.

Scp3 was amplified in all of the PCR reactions, including a small amount in the blank. Mini

preps were performed on the ten colonies in colony PCR 2 (lane 3) in order to obtain the plasmid

DNA from the colonies. Restriction digests with PstI and SalI were used to verify the presence of

scp3 and to determine if scp3 was ligated into pYES forwards or backwards. The possible

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outcomes of the digest are illustrated in Figure 7.

Figure 7. Illustrations showing the cleavage points of Sal I (red) and PstI (blue). Not drawn to scale. A) Scp3 ligated normally. Yields 3 fragments: 3,465, 2,643, and 488 bp. B) Scp3 ligated backwards. Yields 3 fragments: 3,554, 2,554, and 488 bp. C) No scp3 insert. Yields 2 fragments: 488 and 5,398 bp.

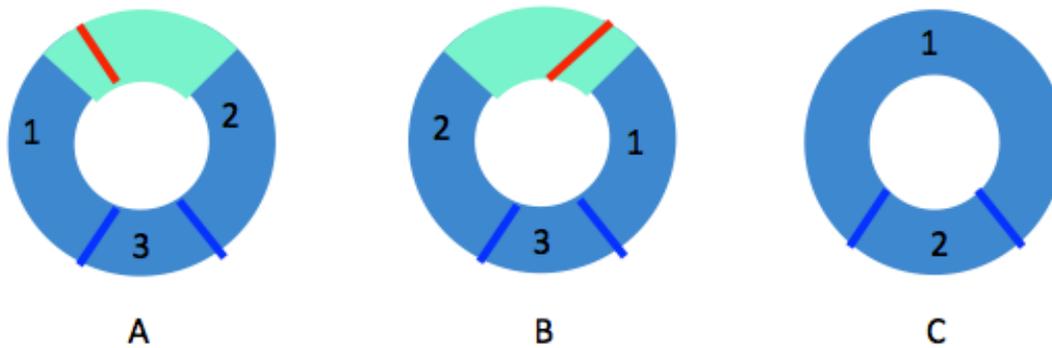The digests were analyzed using 1% agarose gel electrophoresis, shown in Figure 8.
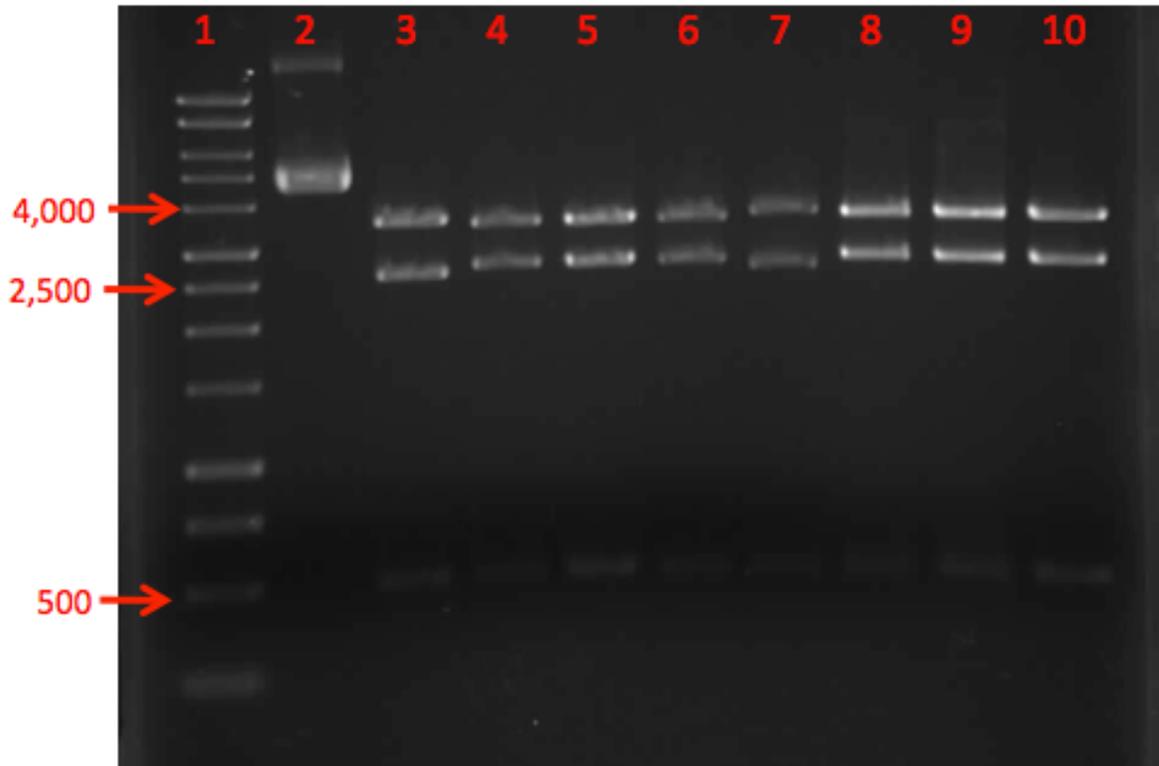
Figure 8. 1% agarose gel showing the restriction digest with PstI and SalI of pYES. Lane 1 is the 1kb molecular weight marker and lane 2 is an undigested mini prep sample. Scp3 is shown ligated forwards in lanes 4, 5, 6, 8, 9, and 10; it is ligated backwards in lanes 3 and 7.

The results of the digest with SalI and PstI showed that scp3 was present in all of the vectors and ligated properly in the plasmids analyzed in lanes 4, 5, 6, 8, 9, and 10.

Verification of the scp3 sequence

Once it was determined that scp3 was ligated into pYES, the vector was sequenced at the SUNY CFG Molecular Core Facility. The sequence of scp3 ligated into pYES matched the sequence of scp3 perfectly, shown by the sequence comparison in Figure 9.

Score	Expect	Identities	Gaps	Strand
1308 bits(708)	0.0	708/708(100%)	0/708(0%)	Plus/Plus

```
Query  1    ATGGAGTCGCATTGTACGGGGAGGAAGCGGGCAATTTGTATCGGCATCGACTACCAAGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGAGTCGCATTGTACGGGGAGGAAGCGGGCAATTTGTATCGGCATCGACTACCAAGGG  60

Query  61   CAGTCGAGTGAGCTGAAGGGATGCATCAACGATGCACGAGACGTGCGCGACTTCCTCGTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAGTCGAGTGAGCTGAAGGGATGCATCAACGATGCACGAGACGTGCGCGACTTCCTCGTC  120

Query  121  AAACGCTGCCGCTTCAGAAAAGAAGATGTCATGCTACTCACCGACGATAAGTCAAGATCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AAACGCTGCCGCTTCAGAAAAGAAGATGTCATGCTACTCACCGACGATAAGTCAAGATCG  180

Query  181  TCGCGGTCTTATCCCTCGCGAGCGAACATGATCAGCGCGATGAAGTGGCTAGTAGAGGGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCGCGGTCTTATCCCTCGCGAGCGAACATGATCAGCGCGATGAAGTGGCTAGTAGAGGGG  240

Query  241  GCGCAAATGCATGATTCGCTCTTCCTGCACTATTCAGGTCATGGCGGGCAAAAACGCGAC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GCGCAAATGCATGATTCGCTCTTCCTGCACTATTCAGGTCATGGCGGGCAAAAACGCGAC  300

Query  301  TTCAACGACGACGAGGCGGATGGGTACGATGAAACTATATTCCCTGTTGACCACGAAGAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTCAACGACGACGAGGCGGATGGGTACGATGAAACTATATTCCCTGTTGACCACGAAGAG  360

Query  361  GCCGGCGTCATCATCGACGATGAAATGCACAAGATCCTCGTCGACCCCCTCCCTGCTGCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCCGGCGTCATCATCGACGATGAAATGCACAAGATCCTCGTCGACCCCCTCCCTGCTGCA  420

Query  421  TGTCGGCTAACAGCCCTATTCGATAGCTGCCATTCGGGAACAGTGCTTGATCTTCCTTAC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TGTCGGCTAACAGCCCTATTCGATAGCTGCCATTCGGGAACAGTGCTTGATCTTCCTTAC  480

Query  481  ATTTATTCTCAACACGGTCGCCTCCGTGGCTCGCATGTCAGCCCTCGTGCGCGGCTTAGG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ATTTATTCTCAACACGGTCGCCTCCGTGGCTCGCATGTCAGCCCTCGTGCGCGGCTTAGG  540

Query  541  CAAGCGTCTCCTGCCGACGTCATCACCTGGTCCGCGTGCAGAGATGGACAAACATCCGTC  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CAAGCGTCTCCTGCCGACGTCATCACCTGGTCCGCGTGCAGAGATGGACAAACATCCGTC  600

Query  601  GATACCTTCGCAGGCGGTGAAGCCGTCGGCGCCATGAGCTACGCCTTCCTCAAAATCCTT  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GATACCTTCGCAGGCGGTGAAGCCGTCGGCGCCATGAGCTACGCCTTCCTCAAAATCCTT  660

Query  661  AGGAATGAGAGGGACTTGTCGTATAGCGACCTGCTGAAGCGCCTACGG  708
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  AGGAATGAGAGGGACTTGTCGTATAGCGACCTGCTGAAGCGCCTACGG  708
```

Figure 9. Nucleotide sequence of scp3 (Query) compared with the sequence in pYES (Sbjct) using Clustal W.[26]

While there were no errors in the scp3 sequence, it was determined that there was a stop codon (TGA) in front of the start codon (ATG) of scp3. This error was introduced through the primers. The beginning sequence including the stop and start codons is shown in Figure 10.
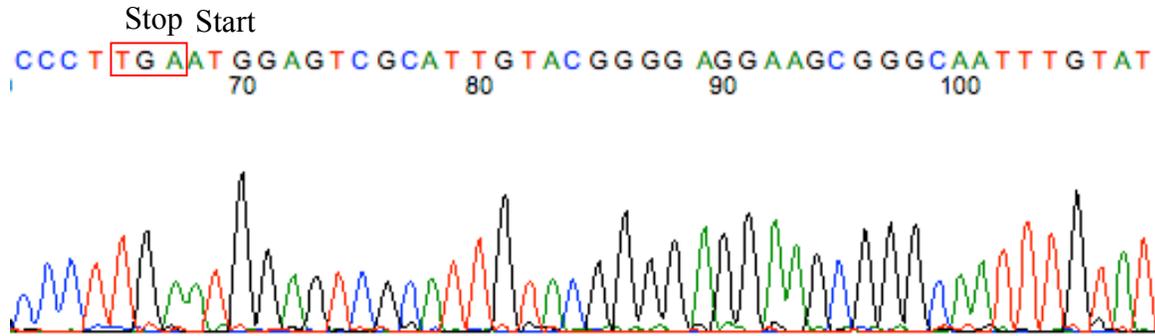
Figure 10. The beginning sequence of scp3 in pYES showing the stop codon in front the start codon (boxed in red).

Transformation into yeast and expression

pYES2.1/V5-His/lacZ, the expression control plasmid containing β-galactosidase, was

transformed into yeast and β-galactosidase was expressed using a galactose medium. The

western blot in Figure 11 shows the expression of β-galactosidase from the kit-supplied
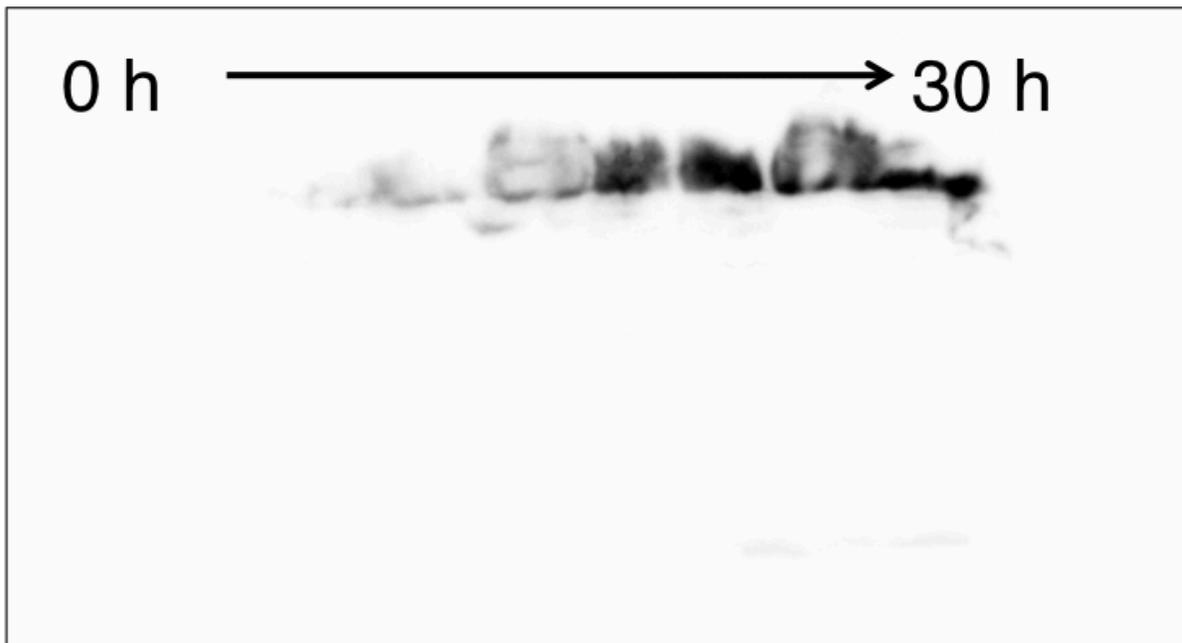
expression control vector.

Figure 11. Western blot of the expression of β-galactosidase in yeast using the expression control vector. The V5 antibody was used for detection.

β-galactosidase expression was monitored for 30 hours, with strong expression occurring after at least 12 hours. The expression of β-galactosiadase shows that the methods developed to insert a plasmid into yeast and express the target protein were successful. This indicates that the inability to obtain Scp3 expression may be due to the mutation in the plasmid, not in the expression procedures that were being used.

CONCLUSIONS AND FUTURE WORK

In order to remove the stop codon, mega primer site-directed mutagenesis was attempted. The goal was to mutate the G in the stop codon (TGA) to a C, creating a serine (TCA). This method was unsuccessful; I was unable to ligate the mutated scp3 into the vector. Instead, the ends of the cut vector ligated together. Future work will be to use an alternative method of mutagenesis using the Q-5® Site-Directed Mutagenesis Kit (New England Biolabs, Inc.). Similar to mega primer mutagenesis, this method utilizes PCR. One primer contains a base-pair mismatch allowing the change from G to C in the sense strand of the plasmid to be perpetuated as the DNA is amplified. The primers sit back to back on the plasmid so that during the PCR, the entire plasmid is replicated. The ends of the plasmid are then ligated while DpnI digests the template DNA.

While Scp3 has not yet been expressed in yeast, we are very close. I have developed a method for successfully expressing protein in yeast. The next step will be to remove the stop codon from the plasmid and then test the expression of Scp3.

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APPENDIX

YPD: Yeast Extract Peptone Dextrose Media
1% Yeast Extract
2% Peptone
2% Dextrose (D-glucose)
To make 1 L:
1. Dissolve 10 g yeast extract, 10 g peptone, and 20 g dextrose in 1000 mL DI water.
2. Autoclave on liquid cycle
3. For liquid medium, store at room temperature or in 4˚C refrigerator.

If making plates,
Add 20 g agar and only add 900 mL of DI water to the YPD mixture before autoclaving. Do **not** add dextrose. Instead, prepare a 20% stock solution of dextrose and autoclave or filter sterilize separately. After autoclaving the YPD broth, add 100 mL of the 20% dextrose. (This is because autoclaving agar and dextrose together will cause the dextrose to carmelize) Let cool before pouring the plates.

Uracil Dropout Media
0.67% yeast nitrogen base (without amino acids and with ammonium sulfate)
2% carbon source (i.e. glucose or raffinose)
0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil)
0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine)
2% agar (for plates)

Make 100X solutions of arginine, lysine, threonine, histidine, isoleucine, methionine, phenylalanine, serine, valine, and proline.
Make 50X solutions of adenine, leucine, tryptophan, and aspartic acid.
Add tyrosine and cysteine last, as solids.

To make 1 L, add 10 mL of the 100X solutions, and 20 mL of the 50X solutions. Dissolve the yeast nitrogenous base and tyrosine and cysteine, bring to 900 mL. Add the agar last if making plates.

**The stock amino acid solutions may be stored at RT
**To make the induction/expression medium, use galactose instead of glucose

Preparation of Sheared Salmon Sperm DNA
Protocol from *Current Protocols in Molecular Biology*
1. Dissolve 10 mg (0.01 g) salmon sperm DNA salt in 1 mL sterile Barnstead water by vortexing. (This is difficult to do because the consistency is like cotton. Had to wear gloves and tear with fingers)
   2. Shear with a 17-gauge needle (we may only have a 22-gauge)

3. Place in boiling water bath for 10 minutes, then chill.
4. Aliquot into multiple sterile 1.5 mL microfuge tubes and freeze at -20˚C.
5. Warm before pipetting.

1 Step Buffer for Yeast Transformation
Protocol from "One step transformation of yeast" for *Current Genetics* 1992
0.2 M Lithium Acetate
40% PEG
100 mM DTT
Sterile Barnstead water

Can make a 1 M stock solution of Lithium Acetate, a 50-70% PEG stock solution, and a 1 M DTT stock solution.

To make 5 mL:
1 mL of 1 M LiAc
2.86 mL 70% PEG
500 µL 1 M DTT
640 µL sterile Barnstead water

10X LiAc (1M Lithium Acetate), pH 7.5
1. For 100 ml, dissolve 10.2 g of lithium acetate in 90 ml of deionized water.
2. Adjust pH to 7.5 with dilute glacial acetic acid and bring up the volume to 100 ml.
3. Filter sterilize and store at room temperature.

Breaking Buffer
50 mM sodium phosphate, pH 7.4
1 mM EDTA (omit EDTA if using this buffer for purification on metal-chelating resins)
5% glycerol
1 mM PMSF

0.1 M Sodium Phosphate, pH 7.4
Sodium phosphate, monobasic (NaH2PO4·H2O; Sigma-Aldrich S9638)
Sodium phosphate, dibasic (Na2HPO4·7H2O; Sigma-Aldrich S9390)

1. Prepare 100 ml of 1 M NaH2PO4·H2O by dissolving 13.8 g in 90 ml of deionized water.
   Bring volume up to 100 ml. Filter-sterilize.
2. Prepare 100 ml of 1 M Na2HPO4·7H2O by dissolving 26.81 g in 90 ml of deionized water.
   Bring volume up to 100 ml. Filter-sterilize.
3. For 1L of 0.1 M sodium phosphate, pH 7.4, mix together 22.6 ml of 1 M NaH2PO4 and 77.4
   ml of 1 M Na2HPO4. Bring up the volume to 1 L with deionized water.
4. Filter-sterilize and store at room temperature.

Yeast Transformation
"One Step Transformation of Yeast" from *Current Genetics,* 1992

1. Prepare an overnight liquid culture of yeast in 10 mLYPD in a 50 mL sterile conical tube, shaking at 30˚C.
2. Fill 1-3 sterile 1.5 mL centrifuge tubes with the liquid culture using a sterile transfer pipette.
3. Spin for 2 minutes at maximum speed.
4. Remove supernatant and resuspend in in 100 µL 1 Step Buffer
5. Add 300-900 ng of plasmid DNA (may experiment with other amounts), plus a blank using sterile Barnstead water instead of DNA
6. Add 5.3 µL salmon sperm DNA (10 mg/µL)
7. Vortex and incubate in a 45˚C water bath for 30 minutes.
8. Plate 100 µL on a uracil dropout agar plate, incubating at 30˚C for 3-4 days

4-6 Protein Expression in Yeast with pYES
pYES2.1 TOPO® TA Expression Kit, Invitrogen, 2004

1. Prepare two 10 mL liquid cultures with glucose uracil dropout medium in sterilized 50 mL Erlenmeyer flasks covered in foil. Shake at 250 rpm in a 30˚C shaker for about 36-40 hours.
2. Determine the OD_{600} of the culture and calculate the amount needed to create an OD_{600} of 0.4 when diluted in 50 mL of induction medium

   Example:

   $$\frac{(0.4 OD/mL)(50mL)}{3 OD/mL} = 6.67 mL$$

3. Remove the amount of culture determined in step 2 and centrifuge the cells at 1500 x g or 4.4 x 1000 rpm for 5 minutes at 4˚C and remove the supernatant.
4. Gently resuspend the pellet in 1-2 mL of induction medium (galactose uracil dropout)
5. Add resuspension to 50 mL induction media in a 500 mL Erlenmeyer flask, shaking at 250 rpm in 30˚C.
6. Take samples ranging from 0 to 30 hours after inoculation into the induction media.
7. At each time point, determine the OD_{600}. Remove 5 mL of culture and centrifuge at max speed for 5 minutes at 4˚C.
8. Remove the supernatant and resuspend the cells in 500 µL of sterile Barnstead water.
9. Transfer the cells to a sterile 1.5 mL centrifuge tube and centrifuge for 30 seconds. Remove the supernatant and store at -80˚C.

Preparing Yeast Lysates
1. Resuspend pellets in 500 µL breaking buffer. Spin at 1500 x g for 5 minutes at 4˚C. Remove the supernatant.
2. Resuspend the cells in a volume of breaking buffer to obtain an OD_{600} of 50-100 (determined earlier)
3. Add about 0.2 g glass beads. Vortex for 30 seconds followed by 30 seconds on ice. Repeat four times.
4. Centrifuge the samples for 10 minutes at maximum speed.
5. Remove the supernatant and put it in a sterile microfuge tube.
6. Determine the protein concentration in the lysate using BSA as a standard.
7. Add SDS-PAGE sample buffer to a final concentration of 1X
8. Load 20 µg of lysate onto SDS-PAGE gel. Then do western blot.
   **Amount of protein loaded on gel not 100% certain. 20 µg may be too much for the control. Do not need to determine protein concentration every single time, just until you have a good idea of how much protein to expect

   **V5 epitope and His tag add about 5kDa to the protein