Examining Human Exocyst Mutations in Model Yeast Cells

A Major Qualifying Project submitted to the Faculty of

Worcester Polytechnic Institute

In partial fulfillment of the requirements for the degree in Bachelor of Science in

Biology and Biotechnology

Written By:

Brittany Jette

Advised by:

Mary Munson, PhD (University of Massachusetts Medical School)

Luis Vidali, PhD (Worcester Polytechnic Institute)

Abstract

The exocyst complex is an eight-subunit protein complex that assists in vesicle trafficking, specifically in the tethering of vesicles to the plasma membrane. Vesicle trafficking is critical to the exocytosis pathway, and cell polarity, which is responsible for guiding the cell to grow and develop properly. Mutations in the exocyst complex have been observed in humans and have been correlated with various diseases, from neurological disorders to early developmental disorders and more. This project investigates the previously identified human mutations in the exocyst complex and a conserved mutation, the loss of amino acids 429-461 in EXOC3 that cause an increase in HDL cholesterol, that is modelled in *S. cerevisiae*. Through cloning and the plasmid swap strategy, the mutant yeast strain demonstrated that the deleted residues were essential for the yeast to survive. Modelling mutations in yeast will help gain a more complete understanding of the structure and function of the exocyst complex and could assist in elucidating how the mutation affects human cell function and disease.

Table of Contents

Acknowledgements

- Dr. Mary Munson for her guidance and support in this project and as my advisor.
- Dr. Luis Vidali for generously providing me space in the lab and support as my advisor.
- Dante Lepore and Billie Reneker for assistance with protocols, techniques, and primer creation.
- Dmitry Korkin for his assistance with Tcoffee and protein alignments.
- My family for their endless support and encouragement throughout this project and my college career.
- The COVID-19 Pandemic has posed many challenges for this project since the University of Massachusetts Medical School did not allow undergraduate students into the labs for research. Due to this limitation, I continued working with the Munson Lab on the project, but separately in the Vidali Lab at WPI. Working separately from UMass delayed some of the work and therefore I did not get as much data as expected.

Introduction

Exocytosis is the process where the cell releases cargo inside of a secretory vesicle to the outside of the cell through fusion of the plasma membrane. For the vesicle to fuse to the membrane, membrane proteins such as SNARE proteins are extremely important. This vesicle contains molecules that are meant to be secreted, whether it is for signaling, development, growth, division, or migration. There are three steps required for exocytosis to occur: delivery, tethering, and fusion. Vesicles are delivered to the plasma membrane by actin and myosin. Once the vesicle is brought to the plasma membrane, tethering can take place. Sec4, which is a Rab GTPase, and the exocyst complex are all required for tethering the vesicle to the plasma membrane. The final step is the fusion of the vesicle with the plasma membrane through SNARE proteins both on the plasma membrane, called t-SNAREs, and the vesicle, which are called v-SNAREs (Brennwald & Rossi, 2007). After these three steps are completed, the vesicle fuses with the membrane and the cargo is released. Interruption of this process could lead to the vesicle not being delivered to the right location, incorrect tethering, or the vesicle not fusing. The downstream effects from defects in the exocyst complex include complications in exocytosis such as disruption of vesicle delivery and the incorrect SNARE to exocyst binding.

The Exocyst Complex

The exocyst complex is a hetero-octameric protein that mediates the tethering and delivery of vesicles to the plasma membrane. This complex is evolutionarily conserved in many eukaryotic organisms, such as humans, mice, flies, and yeast. The human exocyst complex subunits have a different nomenclature than other eukaryotic organisms, the human and yeast names for the protein subunits are shown in Table 1.

Table 1. *The human and yeast names for the different exocyst proteins.*

Human	EXOC1	EXOC2	EXOC3	EXOC4	EXOC5	EXOC6	EXOC7	EXOC8
Yeast	Sec3	Sec5	Sec6	Sec8	Sec10	Sec15	Exo70	Exo84

Each protein assembles to tether the vesicle to the plasma membrane. In eukaryotic cells, two subcomplexes are formed with four proteins in each before binding to the vesicle. One of the subcomplexes is composed of Sec3, Sec5, Sec6, Sec8 and the other with Sec10, Sec15, Exo70, and Exo84. Once the complexes bind to the vesicle, they assemble to become the hetero octamer. In yeast cells the exocyst complex interacts with Sec3 and SNARE proteins to initiate the fusion of the vesicle and the plasma membrane (Mei & Guo, 2019).

Exocytosis and Human Disease

Membrane trafficking plays a large role in development of the cell, especially for cell polarity. Cell polarity, or the asymmetric molecular composition of different portions of a cell, guides the cell to grow, divide, and differentiate. This polarity if formed by different concentrations of different molecules inside or outside of the cell. The exocyst subunits are essential for the cell's survival and deletion of the subunits is lethal, except Sec3, however, the yeast cells without the *SEC3* gene are sick and have decreased function (Linklater et al., 2018).

Development of many organisms are controlled by polarity, including human. For a eukaryote to develop correctly, the membrane trafficking system must be operational. In human development, the initial cell is divided based on the molecular concentrations inside the cell and from there the cell begins to divide and become differentiated. The nervous system is one of the first systems to develop in an embryo with the neural tube developing from the middle of the embryo outwards. One end of the neural tube is fused and the other develops a brain. If the

network for polarity is disrupted, the development of an embryo can become impaired and cause disease. When mutations occur in the human exocyst complex, a wide array of disorders are shown based on the subunit that is affected, this can be observed in Table 2.

Table 2. *The human exocyst complex mutations are shown; only point mutations, deletions, truncations, and SNPs were considered.*

Subunit Affected	Mutation	Disease/ Symptoms	Category
EXOC1	NM_001024924.1:c.1330+1646C>T (rs13117307) single nucleotide polymorphism (SNP), change from the C to T	Cervical squamous cell carcinoma (SCC) (Łaźniak et al., 2017)	Cancer
EXOC2	Homozygous variants in affected children from Family 1 [c.1309C>T; p.Arg437*]	Severe developmental delay, dysmorphism, and brain abnormalities; variability associated with epilepsy; and poor motor skills (Van Bergen et al., 2020)	Neurological
EXOC2	Compound heterozygous in the affected child from Family 2 [c.389G>A; p.Arg130His and c.1739T>C; p.Leu580Ser	Severe developmental delay, dysmorphism, and brain abnormalities; variability associated with epilepsy; and poor motor skills (Van Bergen et al., 2020)	Neurological
EXOC3	Loss of residues 429-461	Increase in HDL cholesterol (Lanktree et al., 2015)	Metabolism
EXOC3	Missense mutation in a highly conserved amino acid, p.Leu41Gln	Dandy-Walker malformation (DWM) (Shalata et al., n.d.)	Neurological
EXOC3	Nonsense mutation, p.Arg72*, no protein detected	Dandy-Walker malformation (DWM) (Shalata et al., n.d.)	Neurological
EXOC5	Complete knockout	Congenital ureteropelvic junction (UPJ) (Lee et al., 2016)	Early development disorder
EXOC5	Complete knockout	Polycystic kidney disease (PKD) (Huang & Lipschutz, 2014)	Early development disorder

EXOC6	Homozygous nonsense variant [c.906T4A/p.(Tyr302*)]	Spondyloepimetaphyseal dysplasia (SEMD), multiple joint dislocations at birth, severe joint laxity, scoliosis, gracile metacarpals and metatarsals, delayed bone age and poorly ossified carpal and tarsal bones, probably representing a yet uncharacterized SEMD with laxity and dislocations (Girisha et al., 2016)	Connective tissue and skeletal
EXOC7	Splice variant, c.809–2A>G, (c.809–2A>G)	Brain atrophy, seizures, developmental delay, and in severe cases, microcephaly, and infantile death (Coulter et al., 2020)	Neurological
EXOC7	In-frame deletion, Ser48del	Brain atrophy, seizures, developmental delay, and in severe cases, microcephaly, and infantile death (Coulter et al., 2020)	Neurological
EXOC7	Splice variant and in-frame deletion, LEG Deletion	Brain atrophy, seizures, developmental delay, and in severe cases, microcephaly, and infantile death (Coulter et al., 2020)	Neurological
EXOC7	Missense, Ala523Thr	Brain atrophy, seizures, developmental delay, and in severe cases, microcephaly, and infantile death (Coulter et al., 2020)	Neurological
EXOC8	Deletion of a CT that causes a frameshift mutation from Asp607Ter	Brain atrophy, seizures, and developmental delay with microcephaly and infantile death (Coulter et al., 2020)	Neurological
EXOC8	Complementary DNA change from A794T which causes a mutation in p.E265G	Joubert syndrome (Dixon-Salazar et al., 2012)	Neurological

Neurological disorders are the most prominent amongst the mutations observed in this table. Some of these neurological disorders do not have names, but display similar symptoms such as developmental delay, brain atrophy, epilepsy, and seizures. Others are more prominent

such as Dandy-Walker malformation, which is a deformity that affects the cerebellum that controls motor function (Shalata et al., n.d.). Joubert syndrome is another disease; it causes abnormalities in the brainstem and the cerebellar vermis (Dixon-Salazar et al., 2012). Neurological diseases seem to be associated with subunits EXOC2, EXOC3, EXOC6, EXOC7, and EXOC8.

Other diseases that have been associated with exocyst complex mutations include cervical squamous cell carcinoma, early development disorders, and connective tissue and skeletal abnormalities. Cervical squamous cell carcinoma is a common malignant tumor of the cervix and a mutation in EXOC1 is associated with this type of cancer (Łaźniak et al., 2017). Early developmental disorders such as congenital ureteropelvic junction (UPJ) and polycystic kidney disease (PKD) have been associated with a mutation in EXOC5 (Lee et al., 2016), (Huang & Lipschutz, 2014). UPJ is the partial or total blockage of urine flow from the ureter to the kidney and can be congenital or acquired. PKD is an inherited disease that causes cysts to grow in the kidneys causing the kidneys to lose function over time. Skeletal and connective tissue abnormalities, such as spondyloepimetaphyseal dysplasia (SEMD), which is an overarching name for major abnormalities in the skeletal and dwarfism, is associated with EXOC6 (Girisha et al., 2016).

High-density lipoprotein (HDL) cholesterol is often referred to as the “good” cholesterol since it brings the cholesterol to the liver to be disposed of. Unlike low-density lipoprotein (LDL) cholesterol, HDL cholesterol has been shown to reduce the risk of heart disease and stroke. A mutation in EXOC3 has been associated with an increase in HDL in a group of individuals (Lanktree et al., 2015).

EXOC3 or Sec6 in yeast plays an important role in the tethering process between vesicles and SNARE proteins. Sec9 and Snc2 are SNARE proteins that are involved in the late stages of exocytosis that assist in tethering to the membrane. Sec6 binds directly with the Sec9 and Snc2 to tether the vesicle to the plasma membrane and initiate fusing of the vesicle and plasma membrane (Dubuke, 2015).

Yeast as a Model System

Budding yeast, *Saccharomyces cerevisiae* or *S. cerevisiae*, is widely used in laboratories as a model organism. *S. cerevisiae* doubles quickly and is easy to culture, making it desirable for completing many experiments in a short period of time. Yeast shares evolutionary systems with plants and animals, such as metabolism, protein folding, and vesicular transport (Mohammadi et al., 2015). Yeast cells are also easy to manipulate through homologous recombination to get the stain of interest for different experiments.

The purpose of this project is to review known disease-associated mutations in the human exocyst complex and use *S. cerevisiae* to model these mutations. If the mutation is conserved in both humans and yeast, those mutations are considered for modelling in yeast cells to examine how the mutation impacts the structure and function of the protein.

Materials and Methods

The goal of this project is to review known disease-associated mutations in the human exocyst complex and demonstrate these mutations in yeast cells. In this following section, the methods completed to reach this goal are explained.

Literature review

Over 30 journal papers were reviewed based on if the research focused on the function of exocyst and exocyst mutations that are associated with human diseases. Large chromosomal mutations were disregarded since these mutations cannot be tested in yeast and therefore were not applicable, this shortened the list of mutations that could be tested in yeast. The mutations then needed to be aligned with the yeast protein sequence to determine if the mutations were conserved.

Alignments

The mutations that were on the list were aligned by performing protein alignments through Tcoffee ("Coffee Server"). Tcoffee is a website that compares protein sequences, aligns sequences, give a confidence level for the alignment, and shows the proteins align with one another based on the sequence and then compare confidence level as well as conservation. The sequences for subunit that had human mutations were submitted to the Tcoffee site along with the same subunit for different species (mouse, C. elegans, fly, S. pombe, and most importantly *S. cerevisiae*). These sequences were used from yeastgenome.org. The alignments were compared through Tcoffee and if the conservation been humans and yeast was the same and the confidence was high, the mutations for the project were chosen. The mutants that were not conserved yeast

and humans were removed from the consideration for making the mutants. Mutants that were conserved with at least humans and yeast were considered for making the mutants in yeast.

The program PyMol (PyMol | PyMol.org) was used to observe the mutations that were conserved in both humans and yeast. In PyMol, the mutation was modelled on the yeast exocyst complex which allows for different displays and visualizing the protein complex in 3D.

Cloning mutants

To test the mutation that was determined through alignment, the cloning of the mutant would need to occur. Making the mutants by cloning into the relevant yeast exocyst gene allows for the examination of growth and secretion defects.

Genomic integration of Sec15-PrA into Sec6 plasmid swap MMY204

A *SEC15*-PrA tag was genomically integrated into a copy of *SEC15*. A PrA tag stands for a protein A tag which is a large string of alanine that is placed at the end of a gene, this group of alanine will assist in pulling out the exocyst complex to purify. The genotype for the yeast strain MMY204 that will be used for the plasmid swap strategy is as follows:

MATα *sec6*Δ::KanMX-4 *his3*Δ*1 leu2*Δ*0 LYS2 MET15 ura3*Δ*0* (*URA3 CEN SEC6*)

MATα means that this is a haploid yeast strain with an alpha mating type. A haploid strain is useful because there is only one copy of each gene and once the PrA tag is incorporated into the genome all the Sec15 proteins will have the tag. The genomic copy of *SEC6* was deleted using a *KanMX* cassette, therefore the *sec6Δ::KanMX*. Since the genomic *SEC6* was deleted, the *URA3 CEN SEC6* plasmid had to be maintained because without a functional *SEC6* gene the cells would die. This plasmid also has the *URA3* gene to produce that amino acid, however the

genotype shows from the *his3Δ1 leu2Δ0* that the cells need to be provided with histidine, leucine, lysine, and methionine to survive.

PCR for PrA tag

Before completing the PCR, a mini prep was performed for the pMM1341 bacteria using the Zyppy™ Plasmid Miniprep Kit Protocol by Zymo Research.

The PCR of the PrA Tag with the *HIS3* cassette was performed using the forward primer (F-*SEC15*-ppx-PrA), the reverse primer (R-*SEC15*-PrA), and the template (pMM1341). The template miniprep was diluted 1:100 and added to the master mix of water, 5xHF buffer, 10mm dNTPs, forward primer (10uM), reverse primer (10uM), and phusion taq. The PCR program run was the following: initial denature at 98ºC for 2 minutes, then 35 cycles of 98ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 1 minute and 30 seconds, and finally 72ºC for 10 minutes. The DNA from the PCR was concentrated by using the DNA Clean and Concentrator™-5 by Zymo Research at a 5:1 ratio of binding buffer to PCR product.

Gel Electrophoresis for DNA of PrA tag

All gels were created using 80mL of 1xTBE, 0.8g of agarose, and 8uL of safe gel dye.

Yeast Transformation

The *SEC15*-PrA gene with the *HIS3* cassette was incorporated into the MMY204 yeast strain. The selection for this strain is for *HIS3* on SCD-His plates, which would only allow growth of the cells that were transformed, since they have the *HIS3* cassette.

50mL of the MMY204 yeast strain was grown overnight at 30ºC shaking at 180rpm. The next day the Optical Density (OD) was checked to ensure that it was between 0.2 and 0.8. Once the cells were in this range, they were spun down at 2.8k rpm for 2 minutes in falcon tubes. The

supernatant was removed and washed with 10mL of sterile water and spun down again for the same rpm and duration. The supernatant was removed and washed with 10mL of LiTE (0.1M Lithium Acetate in 10mM Tris pH 8.0, 1mM EDTA) and then spun again at 2.8k rpm for 2 minutes. The supernatant was removed and then the cells were resuspended in 10mL of LiTE and incubated for 1 hour while rocking. After incubating for an hour, the cells were spun down at 2.3k rpm for 2 mins and the supernatant was discarded. The cells were resuspended with LiTE, the volume for the LiTE was calculated using the following Equation 1.

Equation 1. Optical Density x 50mL x (100uL / 2OD units) = uL of LiTE

The volume was added to the cells and were resuspended. 10uL of the PCR product and 20uL of salmon sperm DNA was added to a microcentrifuge tube along with 100uL of the cells and incubated for 15 minutes at room temperature. 700uL of PEG LiTE was added to the microcentrifuge tube and incubated for 45 minutes at room temperature while rocking, then heat shocked at 42ºC for 15 minutes. After the heat shock, the cells were spun down, the supernatant was discarded, the cells were resuspended in 200uL of sterile water, and the cells were plated onto SCD-His plates using sterile beads and was placed in the 30ºC incubator for 2 days. The negative control of MMY204 was plated on the SCD-His plates to ensure that this strain would not grow.

Western Blot for PrA tag

Eight samples from separate colonies were cultured overnight, and the OD 600 was determined. The volume of cells was determined by dividing 2 by the OD, that volume was spun down on high in 1.5mL Eppendorf tubes. The cells were resuspended in 100uL of water then spun down, 100uL of NaOH was added to the cells, incubated for 10 minutes at room temperature, then spun down at 4000 rpm for 2 minutes and the supernatant was discarded. The

cells were resuspended in 100uL 1x SSB (SDS-sample buffer) and heat cells for 10 minutes at 95°C. 15uL of each sample was added to the wells of a 4-12% gradient gel at 200V for 40 minutes. The transfer buffer was prepared using 25mM Tris, 192mM Glycine, and 20% (vol/vol) Methanol. The gel was removed from the cassette and the foot was trimmed off along with the wells and the gel was immersed in transfer buffer for 10 minutes. The filters were soaked in transfer buffer and the gel and membrane were placed between the two filter papers. The cassette was placed in the transfer tank and the transfer buffer was added to the gel ran overnight at 30V. The blot was removed from the cassette and placed in the blocking solution for 10 minutes. The blot was placed in the primary antibody (anti-Sec15 1:1000) and shook for hour. The blot was washed with 50mL of washing buffer (40mL 10xTBS and 360mL of water) for a quick wash, and then three more times for 15 minutes. The blot was then placed in the secondary antibody (anti-rabbit 1:2000) and shook for an hour. The blot was then washed again with 50mL for a quick was and then the three washes were completed again. The western blot was imaged, and the markers were compared to the sample.

Cloning of Sec6 mutant into pRS315 plasmid

pRS315 was chosen as the backbone for the mutant since the plasmid has the *SEC6* is on a *URA3* plasmid therefore for the plasmid swap strategy the selection will be against the *URA3* plasmid and show the function of the mutant plasmid.

PCR for Sec6 mutant

PCR was completed to amplify the two flanking regions of amino acids 469-498 so when the homologous recombination is done the amino acids will be excluded. Four separate PCR's were completed to orient the *SEC6* insert in the plasmid. The PCR details are in Table 3.

Table 3. *PCR reaction information, primers, and template.*

PCR #	Forward Primer	Reverse Primer	Annealing Temp (ºC)	Template	Expected Size	Extension Time
1	-TTGATGGTACTAA GACTTGTCTAACA AAGAGGCAAAAA AACTGG	DLP79 (M13R)	50	pMM1127	~2kb	1min30 sec
2	TTTTTTTGCCTCTT TGTTAGACAAGTC TTAGTACCATCAA GAAAAA	DLP79 (M13R)	50	pMM1127	~2kb	1min30 sec
3	-TTGATGGTACTAA GACTTGTCTAACA AAGAGGCAAAAA AACTGG	DLP80 (M13F)	50	pMM1127	~1.4kb	1min30 sec
4	TTTTTTTGCCTCTT TGTTAGACAAGTC TTAGTACCATCAA GAAAAA	DLP80 (M13F)	50	pMM1127	~1.4kb	1min30 sec

This protocol is similar to the PCR protocol mentioned above.

Gel Electrophoresis for Sec6 mutant

This protocol is similar to the gel electrophoresis protocol mentioned above.

Homologous Recombination to Clone Sec6 Mutation

The yeast strain MMY10, which is a yeast strain that has a wildtype Sec6 and no other plasmids so the homologous recombination could be completed. The two PCR products that made bands from Table 3 were used for the homologous recombination and would have the selection of *LEU2* on SCD-Leu plates. The schematic for the protocol is shown in Figure 1.

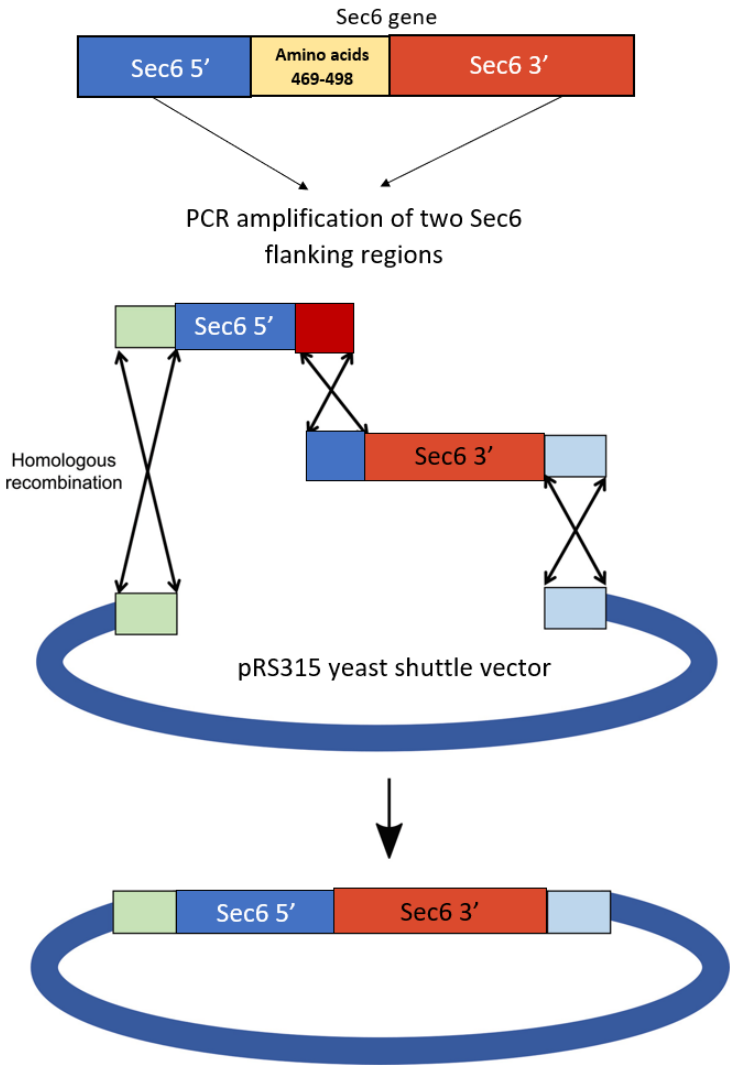

Figure 1. *PCR and homologous recombination were employed to create the mutant Sec6 on a single copy centromeric plasmid.*

The process for growing the MMY102 yeast cells is the same as previously stated on pages 13-14. Equation 1 was used to calculate the volume of LiTE that was added to the two microcentrifuge tubes for the mutant Sec6 and the negative control. The mutant *SEC6* microcentrifuge tube contained 10uL of each PCR product that made a band, 3uL of the linearized pRS315, and 20uL of salmon sperm DNA. The negative control microcentrifuge tube contained 3uL of the linearized pRS315 and 20uL of salmon sperm. 100uL of the cells was

added to each tube and incubated for 15 minutes at room temperature, then 700uL of PEG LiTE was added, and incubated for 45 minutes rocking at room temperature. The tubes were then heat shocked at 42ºC for 15 minutes, the cells were spun down, the supernatant was discarded, resuspended in 200uL of sterile water, and plated on SCD-Leu plates with sterile beads. The plates were places in the 30 ºC incubator for 2 days.

Yeast Plasmid Miniprep and Restriction Digest

A plasmid miniprep from the yeast was performed to ensure that the yeast had the correct *SEC6* insert. Eight colonies from the transformation plates were grown individually in 5 mL of SCD-Leu liquid culture overnight at 30ºC in a tube spinner. The next morning, 1.5mL of culture was spun down in microcentrifuge tubes and the supernatant was discarded. 240uL of Qiagen buffer P1 and 250uL of glass beads were added to each tube and vortexed for 5 minutes on high. 250uL of buffer P2 was added and mixed gently for one minute, then 350uL of buffer N3 was added and mixed immediately and centrifuged on full blast for 10 minutes. The supernatants were added to the spin column and the column was spun for 45 seconds on full blast, the flow through was discarded. The column was washed with 750uL of buffer PE and was centrifuged for 45 seconds, the flow through was discarded and the column was spun for an additional 45 seconds. The column was placed in a clean tube and 30uL of buffer EB was added and sit for 1 minute and then spun for one minute. 30uL of competent DH5-alpha *E. coli* was transformed and plated on LB + ampicillin plates and the plates grew overnight at 37ºC. An individual colony from each plate was placed in 5mL of liquid LB + Amp and let grow overnight spinning at 37ºC. The plasmids were mini prepped from the bacteria using the Qiagen kit.

A restriction digest using EcoR1 was performed to determine which transformants had the correct *SEC6* insert. A digest master mix was created using water, 10X CutSMART buffer,

and EcoRI-HF enzyme. 8uL of the master mix and 2uL of each plasmid DNA were combined and incubated for 1 hour at 30ºC. 2uL of 6X loading dye was added and the reaction was run on a 1%agarose gel for 30 minutes at 200V, the plasmids were sequenced using Genewiz and the Sanger sequencing method and the results were analyzed in Snapgene.

Yeast Transformation of Sec6 mutant plasmid

To test if the pMM1519 plasmid, which contains the 469-498 deletion, is important for survival, the MMY204 cells were transformed with the pMM1519 plasmid, pMM1127, and pRS315, as shown in Figure 2. The -Leu plate is lacking leucine, meaning that a yeast strain that does not have the *LEU2* gene present will not be able to survive. The 5-FOA plate is toxic to cells that have a *URA3* gene. If the protein region that was deleted is essential for the cell's survival, the cells would die, if the cells appear to be sick then the function would be negatively affected causing them to grow slower or not at all, and if the cells appear to grow normally the deleted region is not critical to cell's survival. The MMY204 yeast strain with the pMM1127 plasmid will act as the positive control because it has the wild type Sec6 on a *LEU2* plasmid so will grow on 5-FOA plates. The MMY204 yeast strain with the pRS315 plasmid will act as the negative control because it has a *LEU2* plasmid as well as plasmid with Sec6 and *URA3* so will not grow without that plasmid since it needs the copy of Sec6 but will die with *URA3* on the 5-FOA plates. Figure 2 shows the schematic of how the experiment should run and the expected outcomes.

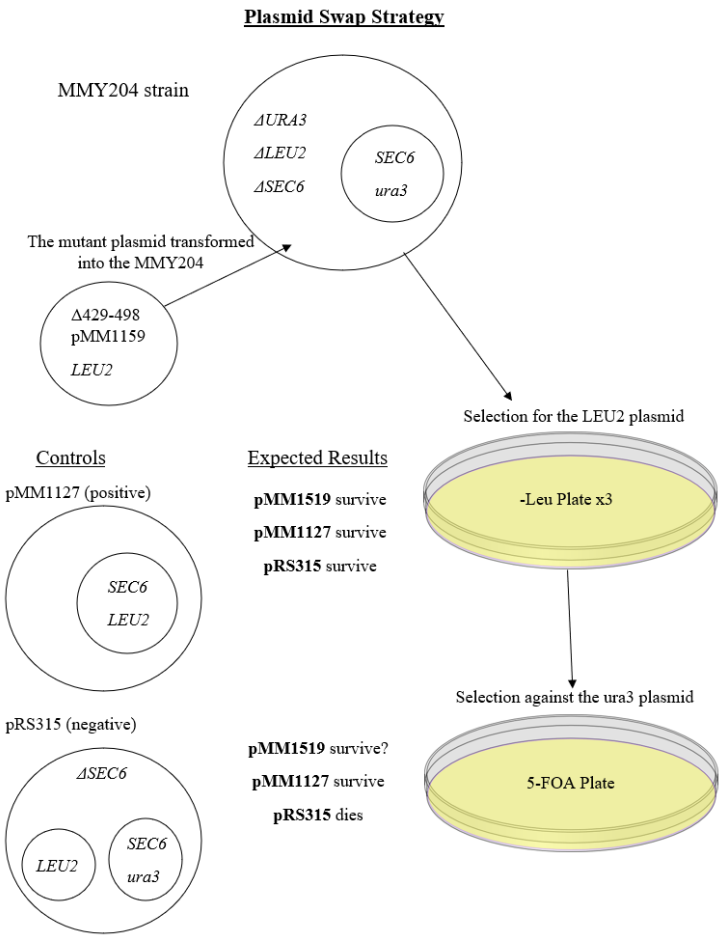

Figure 2. *The schematic for the plasmid swap strategy. The relevant genes are as indicated.*

The process for growing the MMY204 yeast cells is the same as previously stated on pages 13-14. 2uL of plasmid and 20uL of SS DNA were added to labelled tubes and after incubation, the cells were spun down at 2.3k rpm for 2 mins and the supernatant was discarded. The volume of LiTE to add to the cells was calculated using Equation 1. 100uL of cells were added to each tube and incubated for 15 minutes at room temperature, then 700uL of PEG LiTE was added and incubated at room temperature for 45 minutes while rocking. The cells were then heat shocked at 42ºC for 15 minutes, centrifuged, the supernatant was removed, and 200uL of sterile water was added to each tube. All of them were plated on SCD-Leu plates. They grew at 30ºC until the colonies were large enough, the cells were plated on SCD-Leu for three more

times until individual colonies were picked to plate on 5-FOA plates. The conditions for the 5-FOA plating varied from 20ºC, 30ºC, 37ºC to determine if the mutant is temperature sensitive.

Western Blot

The six samples (pMM1519 #1, #2, and #3 as well as pRS315 #1, #2, and #3) were cultured overnight and the same procedure was used as the other western blot. The blot was placed in the primary antibody (anti-Sec6 1:1000) and in the secondary antibody (anti-rabbit 1:2000). The western blot was imaged, and the markers were compared to the sample.

Results

Sequence Alignments

The first mutation that was conserved is Sec5 (EXOC2) with a substitution of a Leucine for a Serine at amino acid 580 in humans and 386 in yeast. This alignment did not show conservation among other species with only the yeast and human sequences being conserved. This mutation in humans causing developmental delay and brain abnormalities. The sequence alignment is shown in Figure 3.

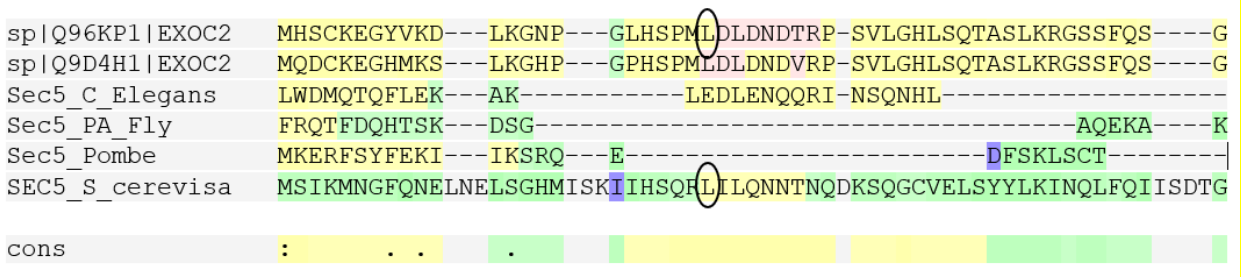

Figure 3. *Protein alignment for Sec5 mutation.*

The second mutation that was conserved is Sec15 (EXOC6B) with a deletion of the Tryptophan at amino acid 302 for humans and 336 for yeast. This alignment was conserved at this amino acid but there was a low confidence in the yeast alignment. This mutation causes skeletal and connective tissue disorders in humans, making this an interesting mutation to see how it affects yeast. The sequence alignment is shown in Figure 4.

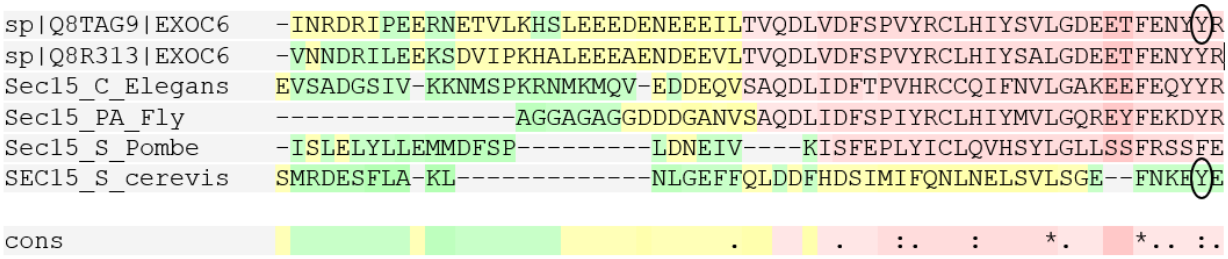

Figure 4. *Protein alignment for Sec15 mutation.*

The final mutant protein is a Sec6 (EXOC3) with a deletion from amino acids 429-461 in the human subunit and from 469-498 in the yeast subunit. This mutation causes an increase in HDL cholesterol in human and the protein sequence is shown in Figure 5.

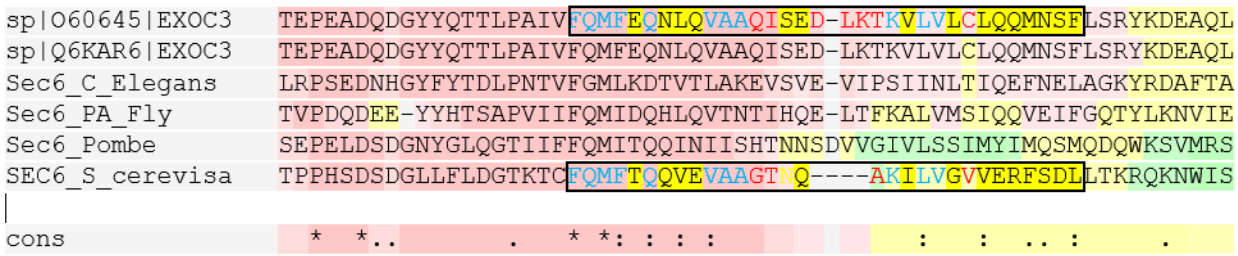

Figure 5. *Protein alignment for Sec6 mutation is shown in the black box. The blue represents conserved proteins, yellow represents proteins that have similar properties such as polarity and the red shows proteins that are not conserved.*

The mutation in Sec6 was chosen for the project since the many of the amino acids were conserved between yeast and humans. The exocyst complex is shown in Figure 6 along with the Sec6 mutation to show the orientation of the mutation relative to the complex.

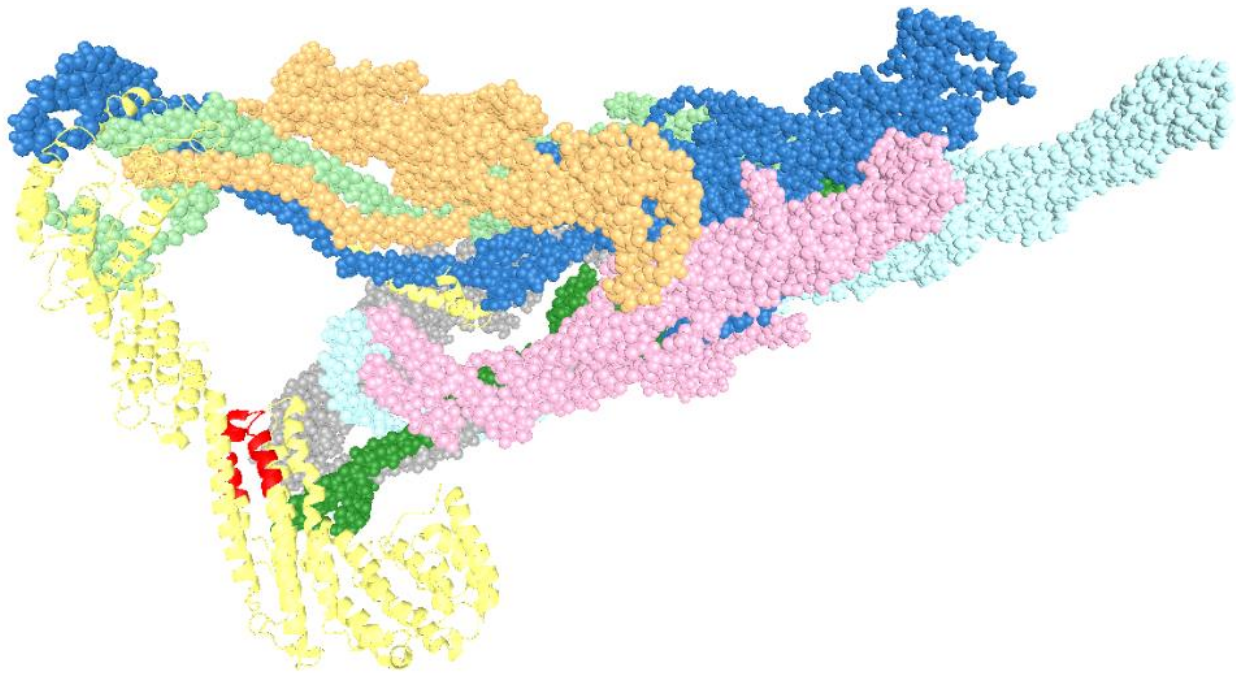

Figure 6. *The full exocyst complex with the SEC6 mutation highlighted in red (PDB code is 5YFP, this shows the cryo-EM structure of the complex). The other subunits are highlighted as followed: Sec3 is orange, Sec5 is light green, Sec8 is dark blue, Sec10 is pink, Sec15 is light blue, Exo70 is grey, and Exo84 is dark green.*

The *Sec6* protein is shown in Figure 7 along with the 469-498 amino acid deletion in relation to the *Sec6* protein.

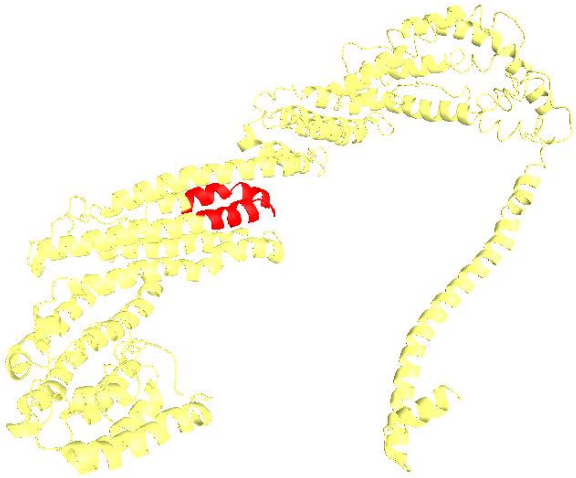

Figure 7. *The Sec6 complex with the 469-498 mutation highlighted in red.*

Integration of PrA Tag into Sec6 plasmid swap strain MMY204

The goal of this gel was to confirm that the PCR product from the PrA tag was the correct integration. Gel electrophoresis results for the amplification of the PCR product for the Sec15-PrA integration shown in Figure 8 indicate that the PCR product that was created with the pMM1341 template and the forward and reverse primers created the properly sized band at ~2000bp.

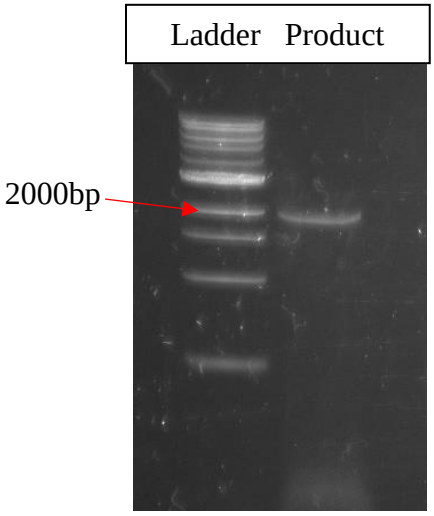

Figure 8. *PrA inserts amplified by PCR.*

The PCR product from Figure 8 was transformed into the MMY204 yeast strain. The goal of this gel electrophoresis was to determine if the transformation was successful and had the proper Sec15-PrA integration. The results for the confirmation of the PrA tag insert is shown in Figure 9. Each sample is from a different colony on the transformation plate, colonies 1-8. The results for this experiment were not as expected, since we expected to observe bands at the ~700bp marker to see the insert. Gel electrophoresis results show that the samples had no bands at the ~700bp mark. This experiment was repeated, and the same results were observed. Although the inserts had not been confirmed, a western blot was conducted anyway.

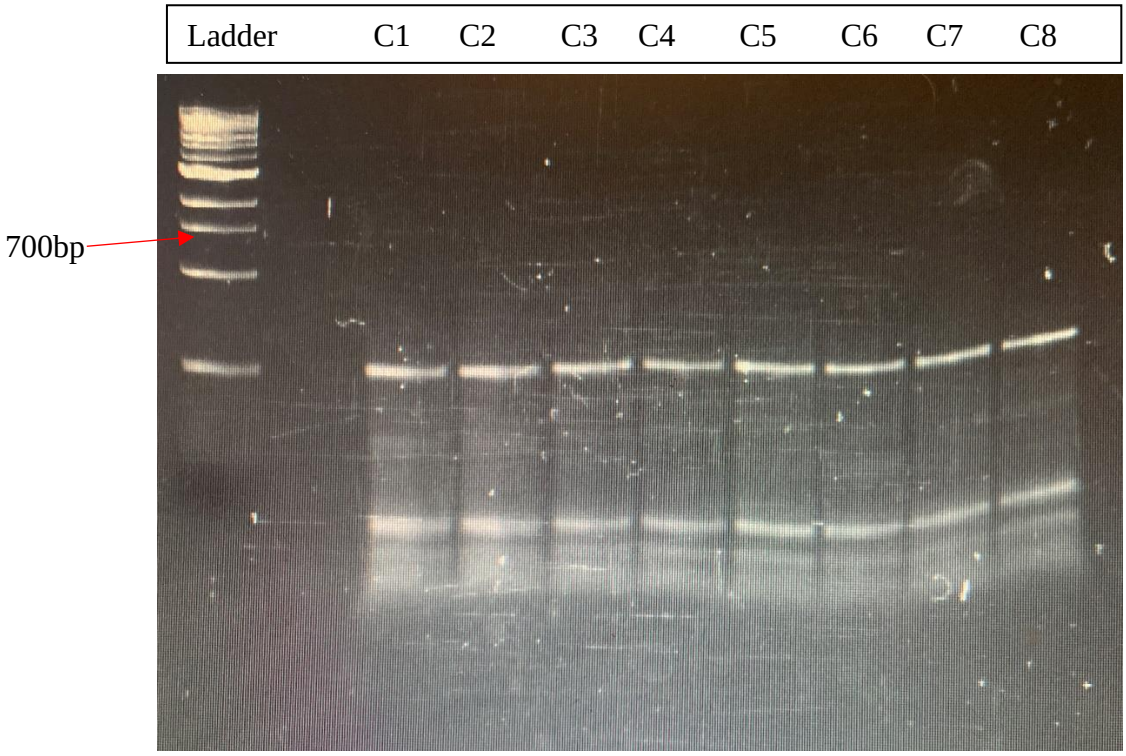

Figure 9. *PrA insert in colonies 1-8. The 700bp band is where the bands were expected to be observed.*

The western blot was completed to confirm the PrA tag that was inserted into the Sec15. The control for the western blot was the wildtype MMY204 yeast strain, the primary antibody was the anti-Sec15, and the secondary was the anti-Rabbit antibody. The western blot results in Figure 10 shows that there is a significant amount of background, which could be from not

washing the assay well enough. The PrA insert band, if integrated correctly, would have been a little higher than 115kDa. The western blot, however, does not show any bands above 100kDa which is where the PrA tag would be located.

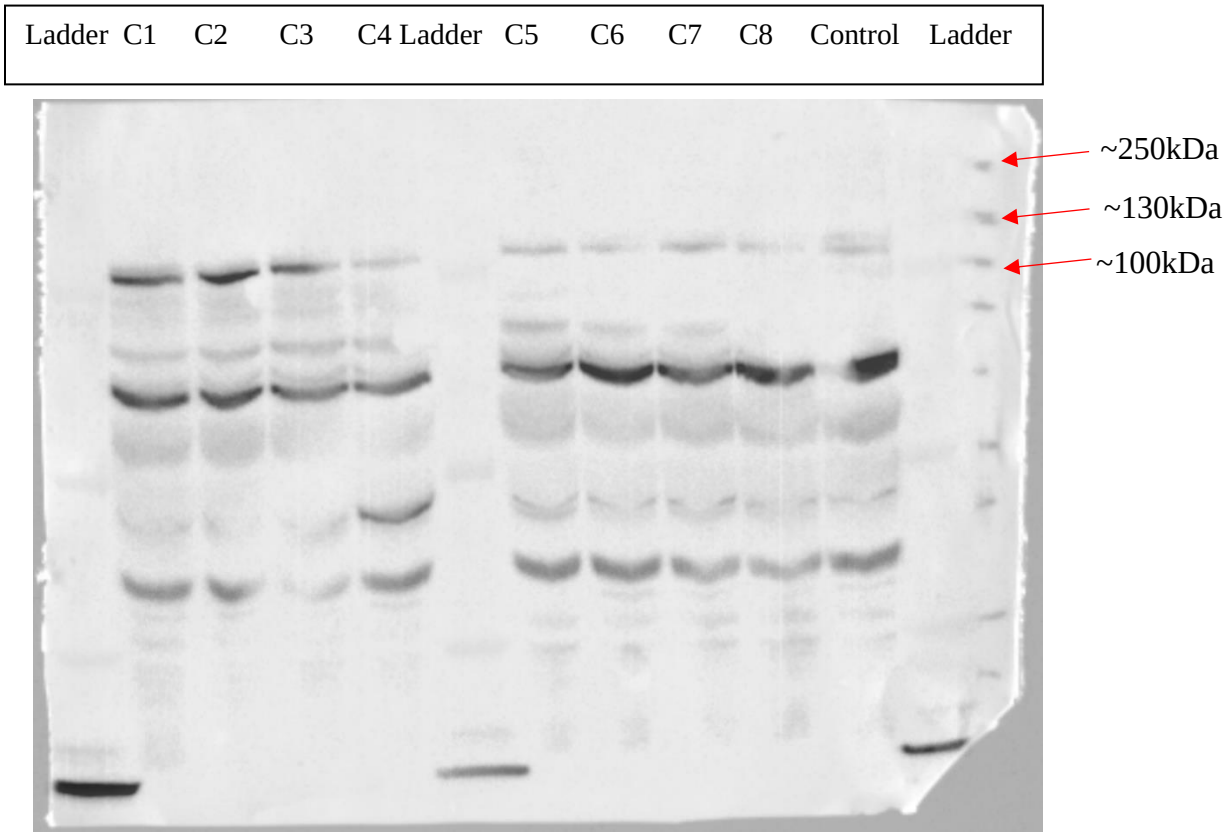

Figure 10. *The western blot for the PrA insert in colonies 1-8, expected band at ~115kDa. The control is the yeast strain MMY204 without the PrA tag.*

Since the MMY204 strain with the pMM1519 plasmid did not survive after transformation, the PrA tag experiments were not redone even though they yielded inconclusive results. Due to the COVID-19 pandemic, there were time constraints that did not allow for more testing for the PrA tag.

Cloning of Sec6 mutant into pRS315 plasmid

The goal of this experiment was to transform the yeast cells to have the mutant plasmid that was created through homologous recombination. Plate results shown in Figure 11 indicate

that the -Leu plasmid is present in the yeast cells as the colonies are growing on SCD-Leu plates and that the control, which is the MMY102 strain without the *LEU2* plasmid is not growing. This confirms that the parent strain does not have this plasmid and that the transformation worked and the yeast cells now have the *LEU2* plasmid.

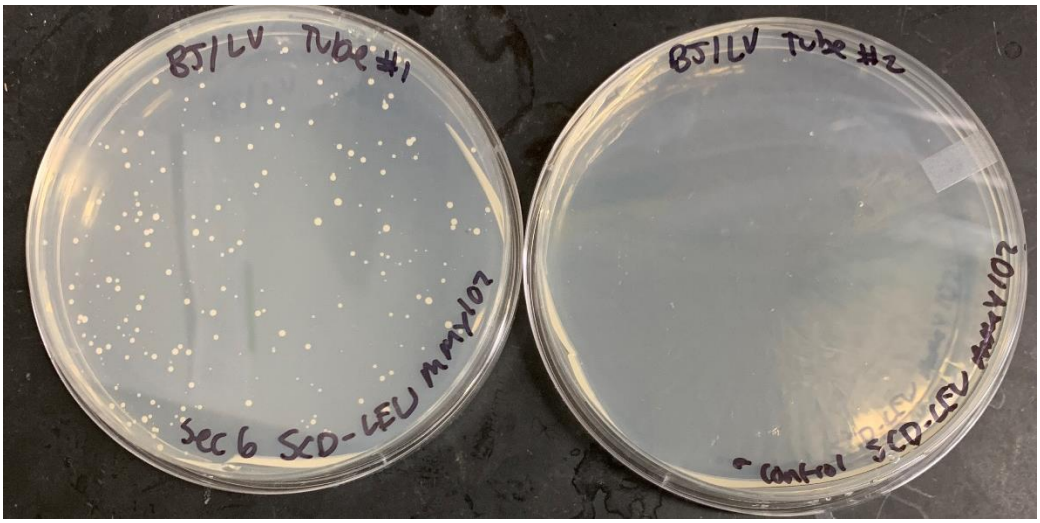

Figure 11. *Transformants for the Sec6 mutant. On the left shows the Sec6 transformants on -Leu plates and on the right shows the control strain, MMY102, on -Leu plates.*

The chosen colonies from the *Sec6* transformant plate were streaked out on -Leu to ensure that they had the *LEU2* plasmid which was expected to have the deletion. The results for this are shown in Figure 12 where the plate on the left is streaked with labelled small colonies that were pulled from the transformant plate and the right plate is labelled with the large colonies. The small and large colonies were chosen to see if they were genetically different since the morphologies were different. The results showed that they were genetically identical.

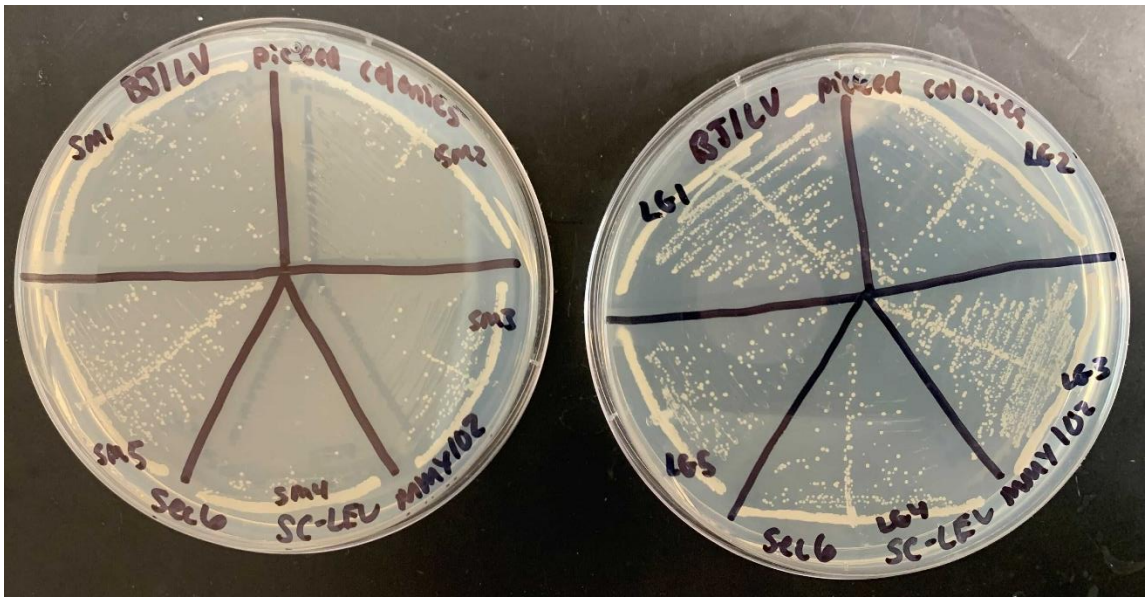

Figure 12. *Colonies picked from the Sec6 plate and streaked out on -Leu plates to get single colonies.*

Six of the transformants from Figure 12 were chosen for plasmid miniprep to confirm if the plasmid was correct. The ones that were chosen were S1, S2, S3, PMM1519, L2, and L3. The PCR product for the chosen mutants is shown in Figure 13. The 500 bp band shown in all the samples if the mutant insert and the other bands are the pRS315 template. Samples S1 and L2 were discontinued at this point since there were other bands present in the PCR that were not expected. pMM1519 was chosen to be sequenced to ensure that the mutant was correct and that there were no other intended mutations. From this point forward only pMM1519 was examined.

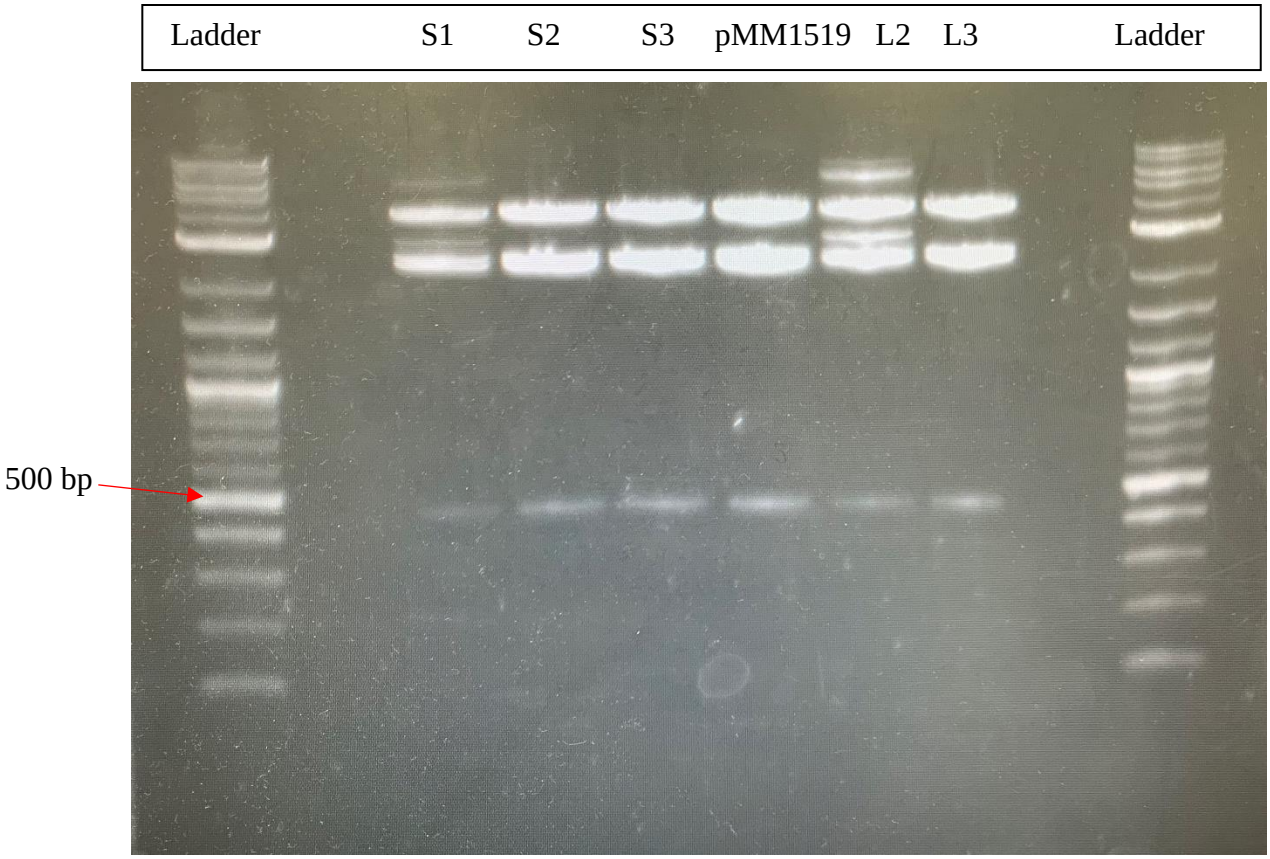

Figure 13. *Sec6 mutants from yeast plasmid rescue.*

Since the sequencing for the pMM1519 plasmid showed that there were no other mutations in the sequence and that the deletion of amino acids 469-498, the MMY204 yeast strain was transformed with pMM1519 as well as the controls. The plating for the pMM1519 transformant as well as the controls are shown in Figure 14. All plates show colonies, which is what was expected since all the strains have the *LEU2* plasmid. Since all the strains had the *LEU2* plasmid, the experiment was continued by streaking the plates out one more time before transferring to 5-FOA. The scheme for this strategy is found in Figure 2.

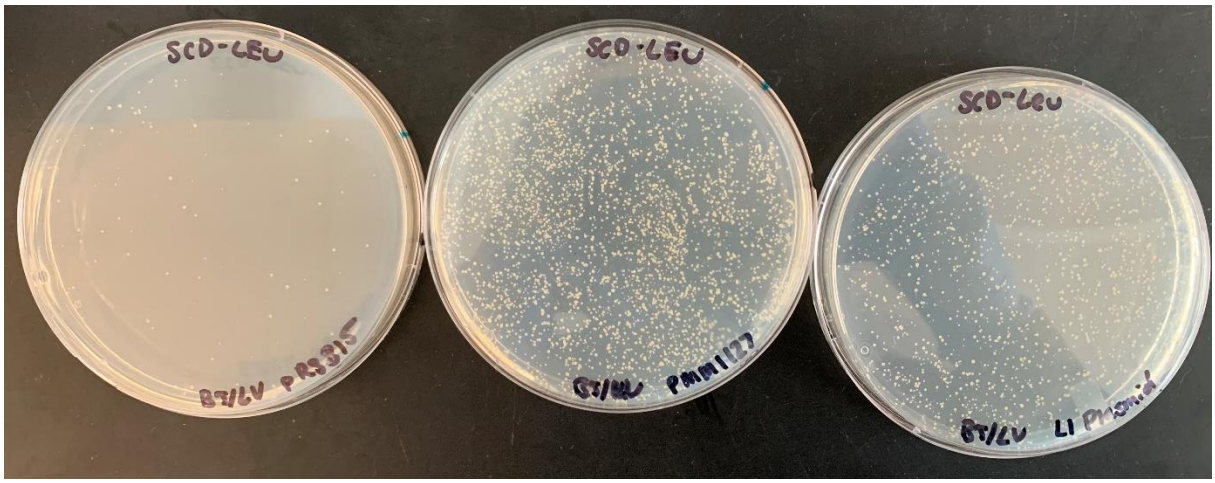

Figure 14. *First transformants for SEC6 mutant transformation. pRS315 is shown on the left, pMM1127 is shown in the middle, and the PMM1519 plasmid is shown on the right.*

The goal of this experiment is to select against the *URA3* plasmid to test if the pMM1519 will produce a functional protein. The MMY204 with the pMM1127 plasmid will be expected to grow since it has the wild type *SEC6* gene and the MMY204 with the pRS315 will be expected to die since the *SEC6* gene is on a *URA3* plasmid which creates a toxic compound that kills the yeast, and the yeast cannot grow without the *SEC6* gene. The results for the transformants plated on 5-FOA are shown in Figure 15. The transformants were tested at different temperatures to test if the mutant protein was temperature sensitive and lead to cell death (or slow growth) at the higher temperature. The results show that the pMM1519 plasmid did not grow on the 5-FOA plate at any temperature. This indicates that the *URA3* plasmid is required for the yeast cells to grow. Since the *URA3* plasmid is providing a wildtype *SEC6* gene, if the wildtype gene is lost and the cells do not survive, that would show that the pMM1519 plasmid is nonfunctional and not able to sustain the cells with the deletion of the amino acids 469-498. There was also growth on plate that had the pMM1127 plasmid, which is normal for that strain since it is does not have a *URA3* plasmid. The plate with the pRS315 plasmid also showed growth on the plate, which

should not have grown. This could be from the plasmid not being the correct plasmid it was labelled as. The YPD shows that the cells would grow normally if they were not being selected for the *URA3* plasmid.

This experiment was redone for accuracy due to the negative control having growth. The results confirmed that the pRS315 plasmid was the correct plasmid and that the new plates worked accordingly. Figure 16 shows the plate results from the repeat experiment and shows that the positive and negative control worked as expected and the pMM1519 did not grow.

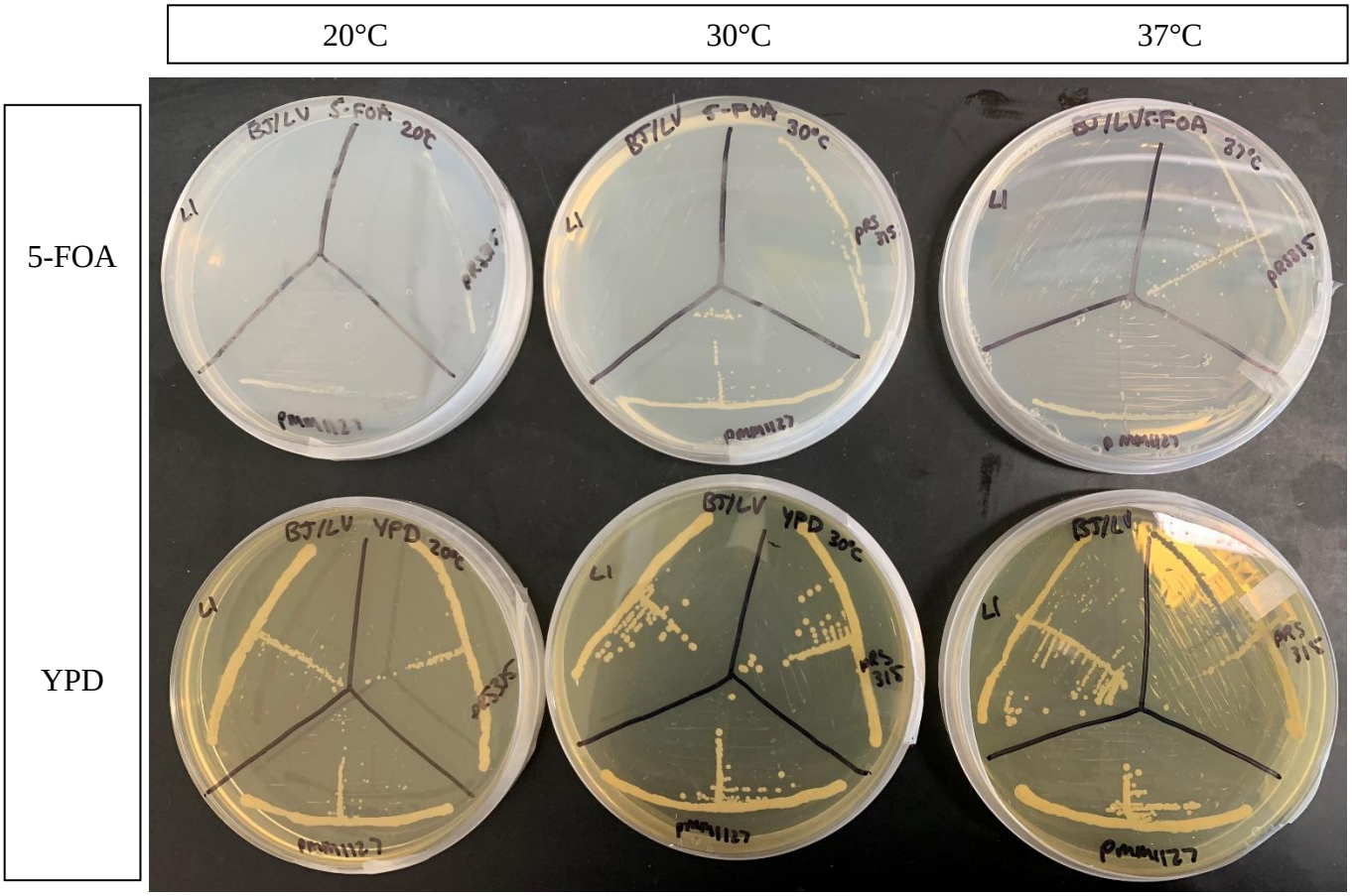

Figure 15. *Each plate shows that yeast cells containing the pMM1519 plasmid, formerly known as L1, is on the left side, pRS315 is on the right side, and pMM1127 is in the middle of every plate for consistency. pMM1519 yeast transformation on 5-FOA at varying temperatures.*

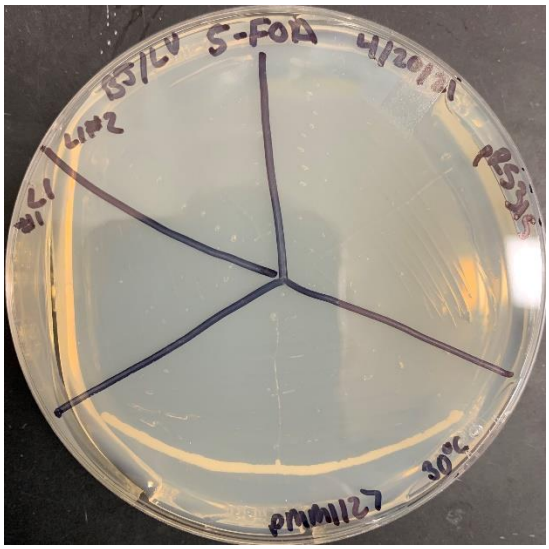

Figure 16. *Repeat experiment of Figure 9.*

A western blot was completed to test whether the pMM1519 plasmid was producing protein or if there was only wildtype protein. Figure 17 shows the western blot which compares the wildtype *SEC6* gene with the pMM1519 plasmid. The results show that there is a significant amount of background protein and bands, suggesting that there could be degradation of the proteins. There is also no difference between the wildtype protein and the pMM1519 protein, suggesting that the pMM1519 is not creating a functional protein and that it may be misfolding. There is also the concern that since the wildtype and mutant protein are so close in size, only 3kb off from one another, that a western blot with this gradient will not allow for proper separation of the two proteins if it is present.

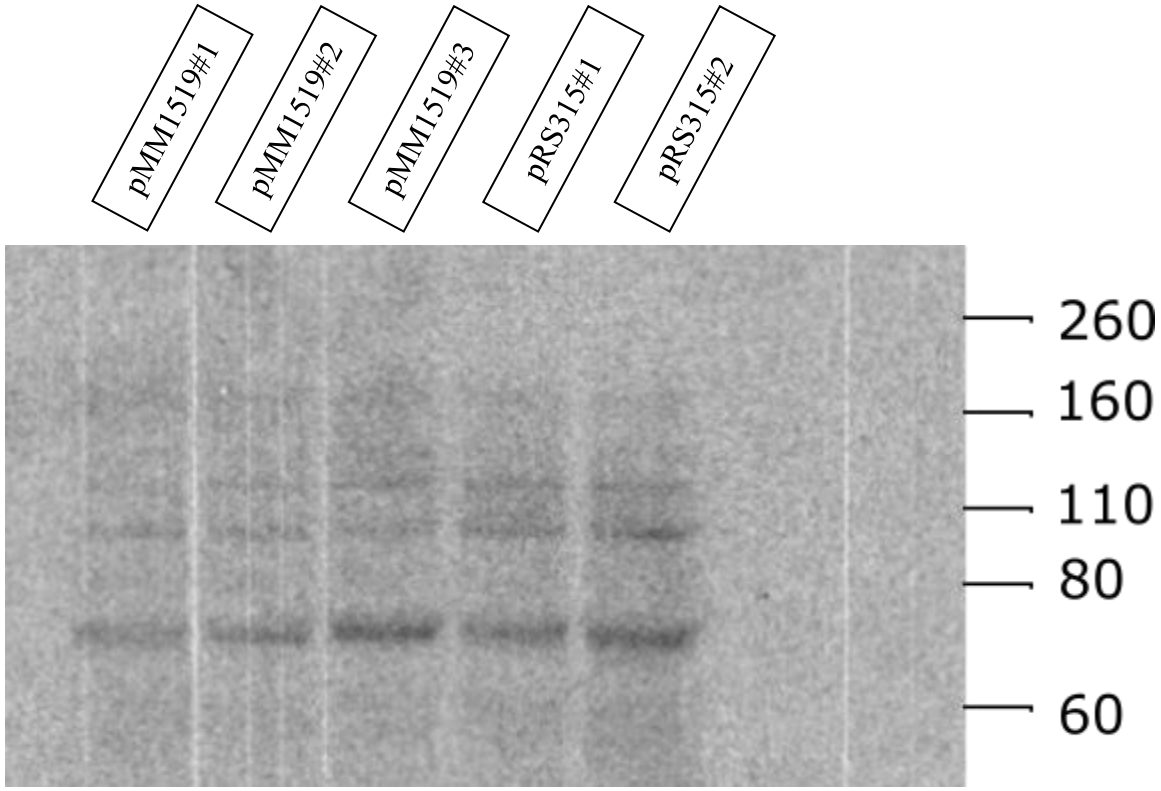

Figure 17. *Western blot comparison of wildtype (control) and pMM1519 Sec6 protein. The expected band for the wildtype protein is ~93kb and the expected band for the mutant protein is ~90kb.*

Discussion

Exocytosis is important for eukaryotic cell development and the exocyst complex is a critical component in the secretory pathway. Mutations in the exocyst complex have been associated with various diseases and effects. By observing mutations in the exocyst complex in the model yeast cells, the impact the mutation has on the structure and function of the complex can be observed. The objectives for this project were to identify and compile a list of mutations in the various exocyst subunits that have been associated with human disease, determine which human exocyst mutations are conserved and cloning the mutants to examine the growth and secretion defects. The mutation that was focused on was the deletion of residues 429-461 in EXOC3 and in yeast it is amino acids 469-498 in Sec6.

The alignments showed that not all the mutations can be modelled in yeast since some are not conserved. The *SEC6* mutation alignment showed that sequence had many conserved amino acids as well as some amino acids with similar properties. The PyMol modelling on the exocyst structure allowed for the visualization of the 469-498 mutation on the structure. Based on the PyMol structure, the mutation is shown on the outside of Sec6 subunit but contributed to two helices as well as a loop. Disruption of these helices could be causing the protein to become unstable, misfold or unfold.

The PrA tagging that was attempted so that the exocyst complex could be purified if the mutation produced live cells was unsuccessful. Since the protein was not functional, the tagging would not have been helpful since even though we could potentially pull out the consequences, there would be a mixture of complexes with the wildtype and mutant protein. This could be a future direction to investigate. Due to time constraints, the PrA tag analysis was discontinued.

The goal of transforming the yeast cells with the pMM1519 plasmid is to observe whether the mutant plasmid would produce living cells and seeing the impact on secretion and function. The transformations of the *SEC6* mutant were successful, however, the cells containing the mutant gene could not survive on its own without a wildtype *SEC6* gene present. Due to the proteins structure and where the mutation is in the protein as shown in Figures 6-7, it seems likely that the mutation is causing the helices to unfold or create a smaller helix that cannot function the way it is supposed to. Sec6 also interacts with SNARE proteins, Sec9 and Snc2, when tethering to the plasma membrane, therefore a disruption in this molecule could cause the inhibition of binding and therefore a stop in exocytosis.

The western blot was completed to compare the protein expression for the pMM1519 and the wildtype Sec6 protein to determine if the yeast cells were producing any protein from the pMM1519 plasmid. The complications that arose with this experiment were that the western blot had a lot of background proteins, and since the deletion and wildtype molecular weights are off by 3kDa, there is not enough separation to be able to see if there is any mutant protein at all. This western blot would need to be performed again with a focus on cleaning the background as well as separating the higher molecular weights which is where the mutant and wildtype Sec6 would run.

For future directions, the other mutations mentioned in Table 2 should be investigated, as well as continuing work with the pMM1519 plasmid. A potential step moving forward with the pMM1519 plasmid would be to make variations of this mutation to see what amino acids are important to the cell's survival and how the amino acids deleted contribute to function. By observing what is occurring when the protein in mutated, we could potentially see why this mutation kills the cells. By characterizing this mutation and observing that it is essential of the

cells to survive, it is interesting to think about how this affects human cells and how the exocyst complex is conserved, but still vastly different since a mutation that causes an increase in HDL cholesterol causes the yeast cells to die due to a non-functional protein. The investigation of more of these mutations could assist in the characterization of more diseases and observing the function of how these diseases occur on a molecular level.

References

Brennwald, P., & Rossi, G. (2007). Spatial regulation of exocytosis and cell polarity: Yeast as a model for animal cells. *FEBS Letters*, *581*(11), 2119–2124. https://doi.org/10.1016/j.febslet.2007.03.043

"Coffee Server." *T*, tcoffee.crg.cat/apps/tcoffee/all.html.

Coulter, M. E., Musaev, D., DeGennaro, E. M., Zhang, X., Henke, K., James, K. N., Smith, R. S., Hill, R. S., Partlow, J. N., Muna Al-Saffar, Kamumbu, A. S., Hatem, N., Barkovich, A. J., Aziza, J., Chassaing, N., Zaki, M. S., Sultan, T., Burglen, L., Rajab, A., … Walsh, C. A. (2020). Regulation of human cerebral cortical development by EXOC7 and EXOC8, components of the exocyst complex, and roles in neural progenitor cell proliferation and survival. *Genetics in Medicine*, *22*(6), 1040–1050. https://doi.org/10.1038/s41436-020-0758-9

Dixon-Salazar, T. J., Silhavy, J. L., Udpa, N., Schroth, J., Bielas, S., Schaffer, A. E., Olvera, J., Bafna, V., Zaki, M. S., Abdel-Salam, G. H., Mansour, L. A., Selim, L., Abdel-Hadi, S., Marzouki, N., Ben-Omran, T., Al-Saana, N. A., Sonmez, F. M., Celep, F., Azam, M., … Gleeson, J. G. (2012). Exome Sequencing Can Improve Diagnosis and Alter Patient Management. *Science Translational Medicine*, *4*(138), 138ra78-138ra78. https://doi.org/10.1126/scitranslmed.3003544

Dubuke, Michelle L, et al. "The Exocyst Subunit Sec6 Interacts with Assembled Exocytic SNARE Complexes." The Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 20 Nov. 2015, www.ncbi.nlm.nih.gov/pmc/articles/PMC4653681/.

Evers, C., Maas, B., Koch, K. A., Jauch, A., Janssen, J. W. G., Sutter, C., Parker, M. J., Hinderhofer, K., & Moog, U. (n.d.). Mosaic deletion of EXOC6B: Further evidence for an important role of the exocyst complex in the pathogenesis of intellectual disability. *AMERICAN JOURNAL OF MEDICAL GENETICS PART A*, 7.

Frühmesser, A., Blake, J., Haberlandt, E., Baying, B., Raeder, B., Runz, H., Spreiz, A., Fauth, C., Benes, V., Utermann, G., Zschocke, J., & Kotzot, D. (2013). Disruption of EXOC6B in a patient with developmental delay, epilepsy, and a de novo balanced t(2;8) translocation. *European Journal of Human Genetics*, *21*(10), 1177–1180. https://doi.org/10.1038/ejhg.2013.18

Girisha, K. M., Kortüm, F., Shah, H., Alawi, M., Dalal, A., Bhavani, G. S., & Kutsche, K. (2016). A novel multiple joint dislocation syndrome associated with a homozygous nonsense variant in the EXOC6B gene. *European Journal of Human Genetics*, *24*(8), 1206–1210. https://doi.org/10.1038/ejhg.2015.261

Huang, L., & Lipschutz, J. H. (2014). Cilia and polycystic kidney disease, kith and kin: Cilia and PKD. *Birth Defects Research Part C: Embryo Today: Reviews*, *102*(2), 174–185. https://doi.org/10.1002/bdrc.21066

Lanktree, M. B., Elbers, C. C., Li, Y., Zhang, G., Duan, Q., Karczewski, K. J., Guo, Y., Tragante, V., North, K. E., Cushman, M., Asselbergs, F. W., Wilson, J. G., Lange, L. A., Drenos, F., Reiner, A. P., Barnes, M. R., & Keating, B. J. (2015). Genetic meta-analysis of 15,901 African Americans identifies variation in *EXOC3PMM1519* is associated with HDL concentration. *Journal of Lipid Research*, *56*(9), 1781–1786. https://doi.org/10.1194/jlr.P059477

Łaźniak, S., Roszak, A., Balcerek, A., Wareńczak-Florczak, Ż., Prokop, E., Sowińska, A., Sotiri, E., Tsibulski, A., Sajdak, S., & Jagodziński, P. P. (2017). Role of rs13117307 single nuclear polymorphism in the risk of uterine cervical cancer from Polish population and its impact on exocyst complex component 1 expression. *Gene*, *627*, 524–529. https://doi.org/10.1016/j.gene.2017.07.012

Lee, A. J., Polgar, N., Napoli, J. A., Lui, V. H., Tamashiro, K.-K., Fujimoto, B. A., Thompson, K. S., & Fogelgren, B. (2016). Fibroproliferative response to urothelial failure obliterates the ureter lumen in a mouse model of prenatal congenital obstructive nephropathy. *Scientific Reports*, *6*(1), 31137. https://doi.org/10.1038/srep31137

Linklater, E., Jewett, C. E., & Prekeris, R. (2018). Polarized Membrane Trafficking in Development and Disease. In *Cell Polarity in Development and Disease* (pp. 121–146). Elsevier. https://doi.org/10.1016/B978-0-12-802438-6.00005-X

Mei, K., & Guo, W. (2019). Exocytosis: A New Exocyst Movie. *Current Biology*, *29*(1), R30–R32. https://doi.org/10.1016/j.cub.2018.11.022

Mohammadi, S., Saberidokht, B., Subramaniam, S., & Grama, A. (2015). Scope and limitations of yeast as a model organism for studying human tissue-specific pathways. BMC Systems Biology, 9(1), 96. https://doi.org/10.1186/s12918-015-0253-0

Shalata, A., Lauhasurayotin, S., Leibovitz, Z., Li, H., Hebert, D., Dhanraj, S., Hadid, Y., Mahroum, M., Bajar, J., Egenburg, S., Arad, A., Shohat, M., Haddad, S., Bakry, H., Moshiri, H., Scherer, S. W., Tzur, S., & Dror, Y. (n.d.). *Biallelic mutations in EXOC3L2 cause a novel syndrome that affects the brain, kidney and blood*. 7.

Van Bergen, N. J., Ahmed, S. M., Collins, F., Cowley, M., Vetro, A., Dale, R. C., Hock, D. H., de Caestecker, C., Menezes, M., Massey, S., Ho, G., Pisano, T., Glover, S., Gusman, J., Stroud, D. A., Dinger, M., Guerrini, R., Macara, I. G., & Christodoulou, J. (2020). Mutations in the exocyst component EXOC2 cause severe defects in human brain development. *Journal of Experimental Medicine*, *217*(10), e20192040. https://doi.org/10.1084/jem.20192040

Wen, J., Lopes, F., Soares, G., Farrell, S. A., Nelson, C., Qiao, Y., Martell, S., Badukke, C., Bessa, C., Ylstra, B., Lewis, S., Isoherranen, N., Maciel, P., & Rajcan-Separovic, E. (2013). Phenotypic and functional consequences of haploinsufficiency of genes from exocyst and retinoic acid pathway due to a recurrent microdeletion of 2p13.2. *Orphanet Journal of Rare Diseases*, *8*(1), 100. https://doi.org/10.1186/1750-1172-8-100