Syracuse University

SURFACE

Spring 5-1-2007

Compensation Mechanisms for Altered Membrane Sterol Compositions in the Yeast: Saccharomyces cerevisiae

David M. DeWolfe

Follow this and additional works at: https://surface.syr.edu/honors_capstone

🌀 Part of the Biochemistry Commons

Compensation Mechanisms for Altered Membrane Sterol Compositions in the Yeast: *Saccharomyces cerevisiae*

David M. DeWolfe

Candidate for B.S. Degree in Biochemistry with Honors

May 2007

Approved

Thesis Project Advisor:_____
Dr. Scott Erdman

Honors Reader:_____
Dr. John Belote

Honors Director:_____
Samuel Gorovitz

Honors Representative:_____

Date:_____

Abstract

Cell membranes are composed of several different lipid and sterol products. Among these are, chiefly, phospholipids, glycolipids, sphingolipids, various proteins posttranslationally modified to carry lipids and sterols. The sterol that is prevalent in fungi, including yeast, is ergosterol. It plays the same physiological role as cholesterol in mammalian cells. That is, mainly, to control membrane fluidity. Membranes in general are extremely important to the normal functioning of any cell and its sub-cellular compartments. The primary factor in the normal functioning of a membrane is the relative composition of the previously mentioned components. Even though there is a high amount of traffic between different membranes within a cell, each one requires its own distinct composition in order to function properly. How cells maintain these distinct compositions is of great interest because abnormal sterol levels have been linked to many diseases in humans, including heart disease and Alzheimer's disease.

In a previous study, a yeast knockout library was screened for sensitivity to a class of anti-fungal drugs called triterpene glycosides. Triterpene glycosides, or TTG, are drugs that work by disturbing membranes. Of the yeast mutants that were found to be hyper-sensitive to TTG, two, *ERG4* and *ERG5*, were found to be involved in the ergosterol biosynthesis pathway. Erg4p, a C-24(28) reductase, is the last enzyme in the ergosterol biosynthesis pathway. When this gene is knocked out, there is a complete lack of ergosterol in the membrane.   Instead, the enzyme's substrate, ergosta-5,7,22,24(28)-tetraen-3beta-ol, accumulates in the membrane. Likewise, when the *ERG5* gene is deleted, the enzyme's substrate, ergosta-5,7,24-trien-3beta-ol, accumulates in the place of ergosterol. The Erg5p is a known C-22 desaturase and immediately precedes C-24(28) reductase in the biosynthetic pathway. Another gene displaying the hyper-sensitive phenotype, *OSH3*, is involved in the transport of sterols to and from the plasma membrane, and the esterification of exogenous sterol products, though its exact function is as yet uncharacterized.

We have begun high copy suppression screens, using TTG, which seek to identify compensation mechanisms between the major components of membranes. Unfortunately, one of these screens did not give enough data to justify continuing the project. The other, however, has been successful, yielding several suppression candidates, and the first phase of the screen is drawing to a close.   Now that suppressors have been found, the lab will then work to understand how these particular genes compensate for alterations in the levels of ergosterol in the yeast membranes and rescue the TTG hypersensitive phenotype. These studies seek to lay the groundwork for understanding the interplay of the various membrane components, the importance of their relative composition in a membrane, and the process by which cells regulate the compositions of membrane components, particularly the primary yeast sterol, ergosterol.

Table of Contents

Acknowledgements

       I would like to thank Dr. Scott Erdman, in whose lab I have worked for the last four semesters, who coordinated my entire project and introduced me to the world of functional genomics research. I would also like to thank Dr. John Belote who, at the last minute, agreed to be my Honors Reader, and whose input on this Capstone Project is invaluable. I would also like to thank the other members of my lab, past and present, for the help, input and advice they have offered during the duration of this project.

Introduction:

The proper distribution of lipids among the cell's various organelles is essential for those organelles' proper functioning. Along with phospholipids, glycolipids, sphingolipids and proteins, ergosterol, a cholesterol like lipid (Figure I-1), is one of the major constituents of yeast cell membranes. Located primarily in the plasma membrane, sterol lipids like cholesterol in mammals and ergosterol in yeast play essential roles in maintaining membrane integrity, structure, fluidity and permeability. Altered sterol composition in these membranes can have far reaching effects on budding, vacuolar fusion, endocytosis and numerous other processes involving membranes and their components (Beh et al. 2001).

The yeast *Saccharomyces cerevisiae*, commonly known as brewer's or baker's yeast, is a simple, single-celled eukaryote that is a commonly used model organism in molecular and cell biology research. While both quickly dividing and easy to maintain, as a eukaryote it shares the complex internal cell structure of plants and animals. This enables the quick application of findings using *S. cerevisiae* to higher organisms. These advantages of using *S. cerevisiae* led to the complete sequencing of its genome, a project completed in 1996. The project resulted in the discovery of over 6200 open reading frames in the haploid yeast's 16 chromosomes and the eventual identification of over 5800 active genes (Goffeau et al. 1996). This advanced knowledge of, and ability to manipulate the yeast genome is what makes

Cholesterol   Ergosterol
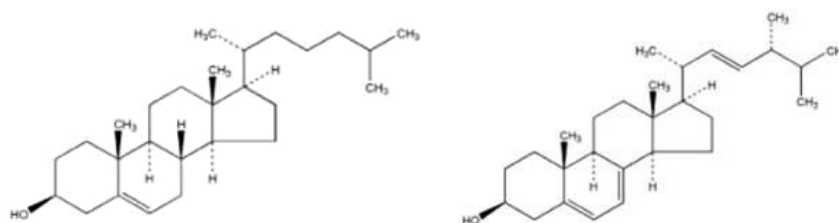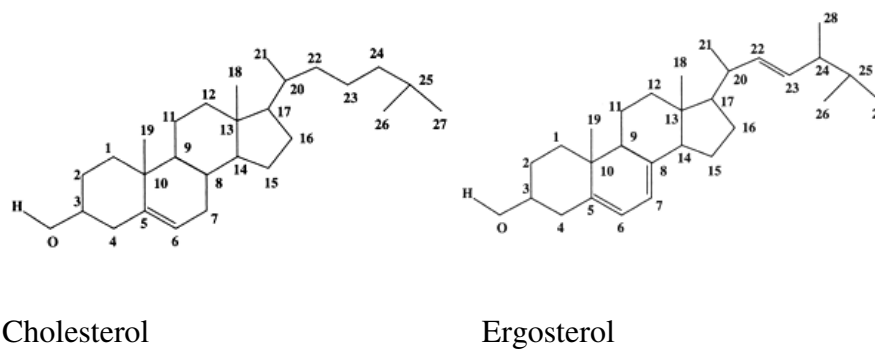

Figure I-1 (www.doctorfungus.org): Cholesterol, the predominant

mammalian sterol is compared here to ergosterol, the predominant yeast

sterol. In ergosterol, we see an additional double bond between carbons 7

and 8, as well as between carbons 22 and 23. Ergosterol also has an

additional carbon, carbon 28, in the form of a methyl group bonded to carbon

24.

S. cerevisiae ideal for understanding the mechanisms by which membrane sterol compositions are maintained.

Used in our research, saponins are a common class of natural antifungal agents found in many plants, including potatoes, oats, tomatoes and cacti, among others. Saponins are amphipathic, with a hydrophilic sugar moiety and a hydrophobic, sterol-like moiety (Figure I-2). It is believed saponins couple with ergosterol to create pores in the plasma membrane of the yeast cells, causing ion leakage as well as disturbing the other functions of the cell's membrane, though the exact mechanism by which saponins act is unknown. However, it is known that the efficacy of saponins is dependent on the presence and levels of ergosterol in the plasma membrane of the target cell (Morrissey and Osbourn 1999).

In a previous study in the Erdman lab, a genetic screen was conducted of the 4,851 viable *S. cerevisiae* deletant (Δ) strains to assess their growth on media containing triterpene glycoside, or TTG, a natural saponin. The goal of this TTG screen was to identify genes involved in lipid homeostasis and to identify genes involved in drug resistance and sensitivity. TTG was selected because of its tendency to intercalate into and disrupt the plasma membrane of fungi based on its interaction with ergosterol (Figure 1-3). Of the 4,851 genes tested, 110 were identified as super-sensitive to TTG. I selected three genes: *ERG4*, *ERG5* and *OSH3*, which are known to have functions related to the sterol composition of yeast membranes on which to conduct further

Hydrophobic Sterol-like Moiety

Hydrophilic Sugar Moiety
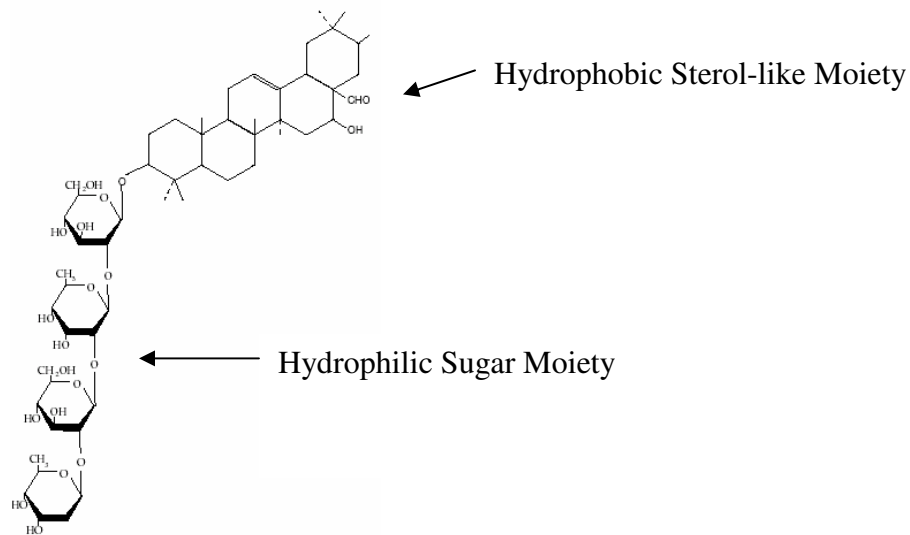
Figure I-2 (http://syllabus.syr.edu/bio/seerdman/TTG/): This is the structure of triterpene glycoside, or TTG, the saponin used in these screens. The hydrophilic sugar moiety consists of four, 6 carbon sugars and the hydrophobic moiety consists of five, 6 carbon rings and is similar in structure to steroids and sterols.

Figure I-3 (Morrissey and Osbourn 1999): This demonstrates the method by which saponins integrate into the plasma membrane, bind with sterols and cause pores to form, through which ions and other necessary cellular molecules may leak.

studies. The method of choice for the study of these genes is high copy
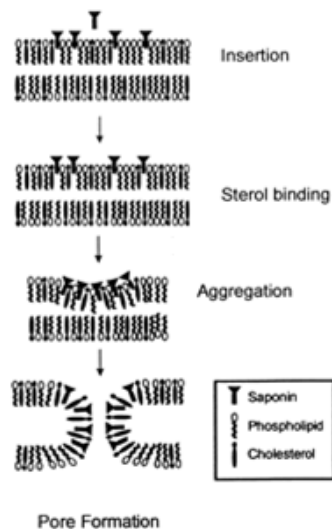
suppression.

High copy suppression is an approach that seeks to find other genes

within the yeast genome that interact with the target gene, i.e. *ERG4, ERG5*

or *OSH3*. The over-expression of certain genes in these deletant strains may

enable them to grow on media containing TTG. The over-expression is

accomplished by transforming the deletant strains with plasmids containing

yeast genes and putting the strains under such conditions that the genes

contained in the plasmids will be expressed. Such genes that do facilitate

growth of the super-sensitive strains are termed "suppressors" and are likely

to have a similar function to the deleted gene, or possibly operate

downstream in the pathway in which the deleted gene is involved.

ERG4 and *ERG5* are genes encoding proteins (Erg4p and Erg5p

respectively) involved in the ergosterol biosynthesis pathway (Figure I-4).

Erg4p corresponds to C-24(28) reductase, the last enzyme in the ergosterol

biosynthesis pathway. When *ERG4* is knocked out, there is a complete lack

of ergosterol in the cellular membranes.   Instead, the enzyme's substrate,

ergosta-5,7,22,24(28)-tetraen-3beta-ol, accumulates in the membrane.

Likewise, when the *ERG5* gene is deleted, the enzyme's substrate, ergosta-

5,7,24-trien-3beta-ol, accumulates in the place of ergosterol. The protein

which the ERG5 gene encodes is C-22 desaturase, which immediately
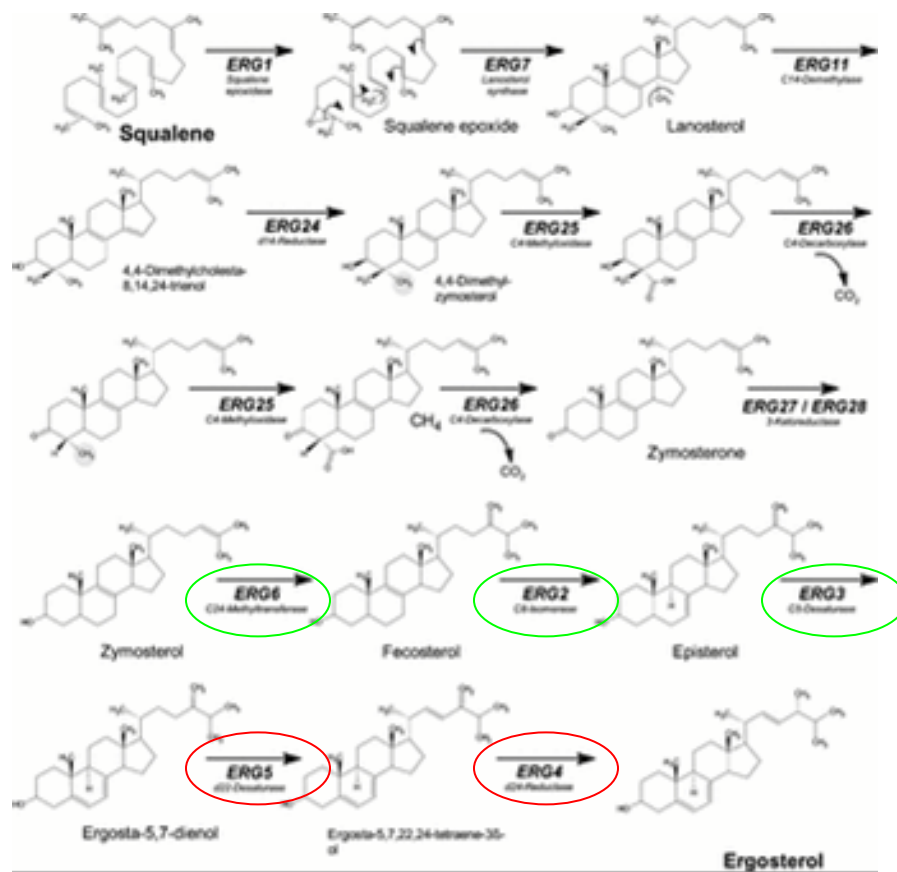
Figure I-4 (www.biochemsoctrans.org): This is the ergosterol biosynthesis

pathway after the production of squalene, a common precursor molecule.

The deletion of *ERG4* and *ERG5*, circled in red, results in a super-sensitive

phenotype. The deletion of *ERG6, ERG2* and *ERG3,* circled in green, results

in a resistant phenotype.

precedes C-24(28) reductase in the ergosterol biosynthetic pathway (www.ihop-net.org).

These genes were chosen for study because it is unknown how their deletion is involved in the increased efficacy of TTG. The situation is especially interesting considering the deletion of the three genes prior to *ERG4* and *ERG5* in the biosynthetic pathway: *ERG3, ERG2* and *ERG6*, resulted in TTG resistant phenotypes (Figure I-4). Because *ERG4* and *ERG5* are so closely related in terms of their place in the ergosterol synthesis pathway, it is likely that many of their suppressors would be the same, and they would also perhaps be suppressors of each other. In order to avoid wasting time, I decided to perform the high copy screen on an *erg4Δ erg5Δ* double deletant strain, and then to test each suppressor on both an *erg4Δ* and *erg5Δ* single deletant strain.

The *OSH3* gene is one of seven oxysterol binding protein homologues present in the yeast genome. While the specific function of *OSH3* is as yet incompletely understood, oxysterol binding proteins are a family of conserved lipid-binding proteins that have been implicated in the maintenance of sterol composition in cell membranes (Beh and Rine 2004). While any one of the *OSH* genes is sufficient for survival, the deletion of the entire family is lethal. This implies the *OSH* family shares in at least one essential overlapping function (Beh et al. 2001). The deletion of any combination of *OSH* genes results in sterol and membrane defects, which

implies a regulatory link between the *OSH* family and sterol lipids (Beh and Rine 2004). The *OSH* family has been directly implicated in the non-vesicular transport of sterols between cellular membranes as well as being involved in regulatory pathways for sterol synthesis (Figure I-5). *OSH3* specifically has been implicated in playing a role in the esterification of exogenous sterols (Raychaudhuri et al. 2006).

The *OSH3* gene is interesting for much the same reasons as *ERG4* and *ERG5* are. The reason *osh3Δ* deletant strains are super-sensitive to TTG is unknown. Also, the deletion of another member of the *OSH* family of genes, *OSH4*, was identified in the initial screen as causing resistance to TTG. In addition, the nature of the role Osh3p plays in the general workings of the cell is still something of a mystery. Using the high copy suppression method, I hope to identify suppressors that may elucidate specific functions of Osh3p as well as explain the nature of its TTG phenotype. Furthermore, this is the groundwork for a better understanding of the mechanism by which cells traffic sterols and maintain membrane homeostasis, which may be of relevance to similar problems in mammalian cells where defects in sterol storage, trafficking, and synthesis may lead to a number of diseases. Cholesterol, the mammalian sterol, is linked to numerous diseases from heart disease to Alzheimer's. Not only high cholesterol levels, but low cholesterol levels as well, are linked to diseases, making the understanding of the

processes by which cells regulate, synthesize, traffic and store sterols of significant importance.
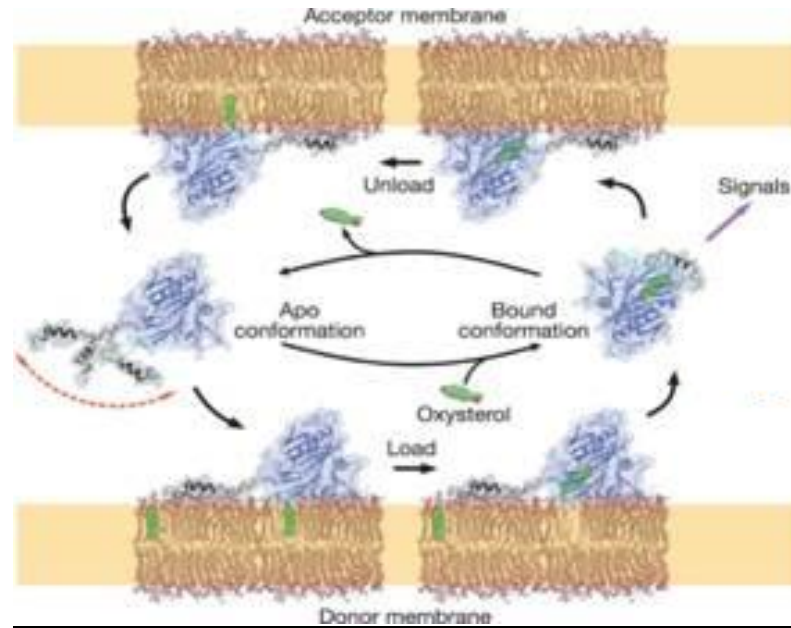
Figure I-5 (Im et al. 2005): This figure depicts Osh4p collecting oxysterols from the donor membrane and transporting them to the acceptor membrane. As the figure shows, Osh4p is also believed to be involved in negative feedback regulation signaling pathways for the regulation of ergosterol biosynthesis. Osh3p is believed to have a similar structure and all the OSH proteins are believed to be capable of this function. Thus, this figure is also a general depiction of the appearance and general function of all the OSH proteins.

Methods and Materials:

Creation of Double Mutants:

A screening strategy first involving the creation of double mutants was designed to make my screen more efficient by eliminating unwanted and uninformative suppressors. As the *ERG4* and *ERG5* genes are so closely related in their place in the ergosterol biosynthesis pathway, it was likely that many of their suppressors would be the same, and that they would likely suppress each other. Performing a single screen on the double mutant strain was like performing two such screens on the single mutant strains. This method takes advantage of the fact that the *ERG4* and *ERG5* genes are on separate chromosomes, chromosomes 7 and 8 respectively. It also takes advantage of the fact that haploid strains can be mated together to form diploid yeast, which in turn will undergo a meiotic division to produce 4 haploid daughter cells. Because *ERG4* and *ERG5* are on separate chromosomes, there is a 50% chance that the meiotic division will produce haploid double mutant strains after sporulation (Figure II-1).

The process begins with mating two haploid strains. Mating type "a" *erg4*Δ cells were combined with mating type "ά" *erg5*Δ cells in YPD (Yeast extract-Peptone-Dextrose medium) liquid medium and grown on a YPD plate. The cells were transferred to a SC-Met/-Lys plate which selected for
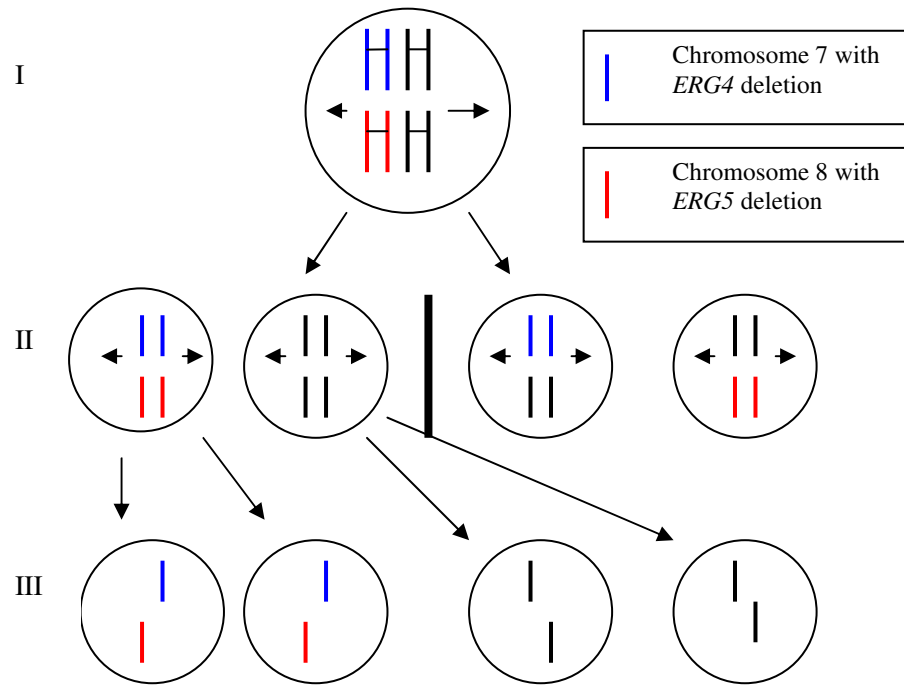
Figure II-1: This figure shows the process by which the *erg4 erg5* double mutant haploids are made. Row I shows the diploid created by mating haploid *erg4Δ* and *erg5Δ* strains. Row II shows the possibilities after the first division of meiosis. On the left side of Row II, both the chromosomes containing the *erg4* deletion, shown in blue, and that containing the *erg5* deletion, shown in red, have migrated into one daughter cell, while the chromosomes without a deletion have migrated to the other. Row III shows the haploid daughter cells resulting from the second meiotic division, where ½ of the daughter cells are double mutants. The right side of Row II shows the other possibility after the first meiotic division, where the chromosomes containing the deleted genes have migrated to opposite daughter cells. Though it is not shown in the diagram, this will result in 4 single mutant haploid daughter cells.

the diploid cells. This is due to the fact that all of the lab's mating type "a" strains are *met15Δ* and all the mating type "ά" strains are *lys2Δ*. Thus, in these crosses, parental mating type "a" cells will be unable to grow due to the lack of methionine and parental "ά" cells will be unable to grow due to the lack of lysine. The only cells able to grow will be diploid cells formed by matings and that contain a single functional copy of both the *MET15* (from the "ά" strain) and *LYS2* genes (from the "a" strain). The diploid cells are then placed in 20% potassium acetate (KAc) solution for 5 days to induce sporulation. Sporulation is the process in the yeast life cycle at which diploid cells divide by meiosis and form an ascus containing four haploid spores, referred to as a tetrad (Figure II-2).

   After sporulation, the tetrads resulting from meiosis are transferred to a YPD plate and dissected using a micromanipulator, which is a microscope fitted with a pulled glass needle used to move individual cells on a plate. Each haploid daughter cell is placed in a line with its three other sister cells and allowed to grow. A replica of this YPD plate is then made on a YPD+Kan (YPD plus Kanamycin, an antibiotic) plate. Once allowed to grow, two growth patterns are observed. In one case, where all four haploid daughter cells are single mutants, all four will grow on the selective plate. In the other case, where two of the cells are haploid double mutants and the other two are wild type with respect to the deleted genes (Figure II-1), only the haploid double mutants will be able to grow. The later case we refer to as
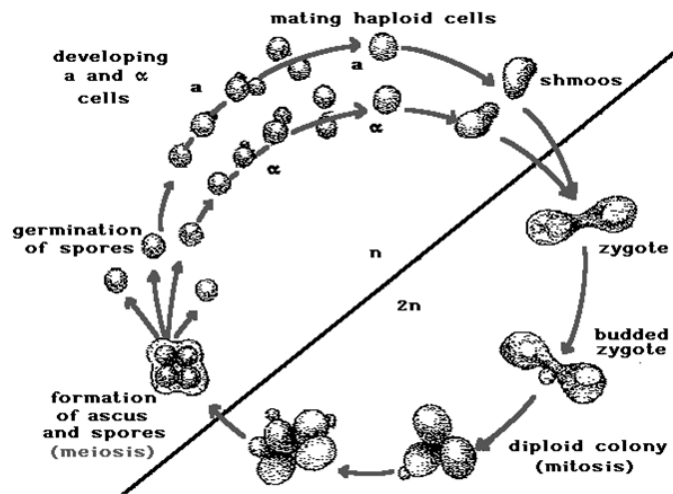
Figure II-2 (http://www.phys.ksu.edu/gene/a2f3.html): This figure shows

the yeast life cycle both as a haploid (n) and diploid (2n).
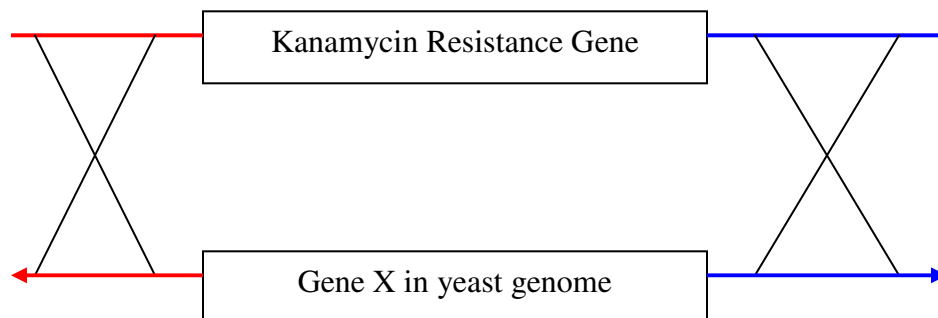
Figure II-3: This figure represents homologous crossover. Yeast cells will

naturally swap regions of highly similar sequence in their genome, color

coded in this figure as red regions and blue regions, with free DNA. This

method was utilized to create the mutant library that served as the basis for

the initial screen of all the viable haploid mutants against TTG. In this

library, the genes were replaced with a gene conferring resistance to

kanamycin. This allows us to select for mutants using kanamycin plates.

growing, "2 and 2" because two will grow and two will not. This is because in this knockout library, the deleted genes have been replaced by homologous crossover with a kanamycin resistance gene (Figure II-3). When haploid double mutants result from meiosis, two of the four daughter cells, those that contain the chromosomes with the kanamycin genes that replaced *ERG4* and *ERG5*, will be able to grow; these are then referred to as double mutant candidates. The other two daughter cells that lack resistance to kanamycin will not be able to grow on the selective plate. The cells are termed "candidates" because, while they fit the growth patterns expected of haploid double mutants, their genetic makeup is not yet confirmed. The yeast genome is very fickle and subject to mutation, especially when placed under stress, so further steps are needed to ensure the growth pattern is due to the genetics we expect. When double mutants do not result, all four of the daughter cells will be able to grow on the YPD+Kan plate, as they all have one gene conferring resistance. The next step is to determine the mating type of the candidate double mutants (**mating type determination**).

Mating type "a" candidates are selected. The preference for mating type "a" cells is arbitrary. However, we pick one mating type and keep it constant throughout the process to keep the genetics as similar as possible across the screen. The mating type "a" candidates are then plated on both SC-Met and SC-Lys plates. Since these haploids are new, it is not guaranteed

that the mating type "a" strains will be *met15Δ*, so they are screened at this

point in order to keep the genetics of the screen as consistent as possible.

The genomic DNA is then collected (**yeast genomic miniprep**) from

the mating type "a" candidates that grow on SC-Lys but not on SC-Met. We

then perform two PCR reactions (**PCR reaction**) on the DNA. One is run

with an ERG4ck primer and KanMX primer and the other with the ERG5ck

and KanMX primers. The PCR products are then run on a .8% agarose gel

(**agarose gel electrophoresis**). A band will appear on the gels at

approximately 700bp if the PCR reaction was successful. A band at 700bp in

both the lanes utilizing the ERG4ck and ERG5ck primers signifies the

candidate is in fact an *erg4Δ erg5Δ* double mutant.

High Copy Suppression Screen

The high copy suppression screen is intended to identify genes that

have a function similar to, or possibly downstream in the pathway of the

deleted gene or genes. The first step in the screen is to create positive and

negative controls, to which the growth of the deletant strains can be

compared. This is accomplished by transforming (**high-efficiency**

transformation of yeast) several strains whose growth pattern on TTG+

media is known with an "empty" plasmid. The pRS315 plasmid, referred to

here as the p19 plasmid, carries the same nutritional marker, LEU2, as the

plasmid used to transform the deletant strains (Figure II-4) except that it does

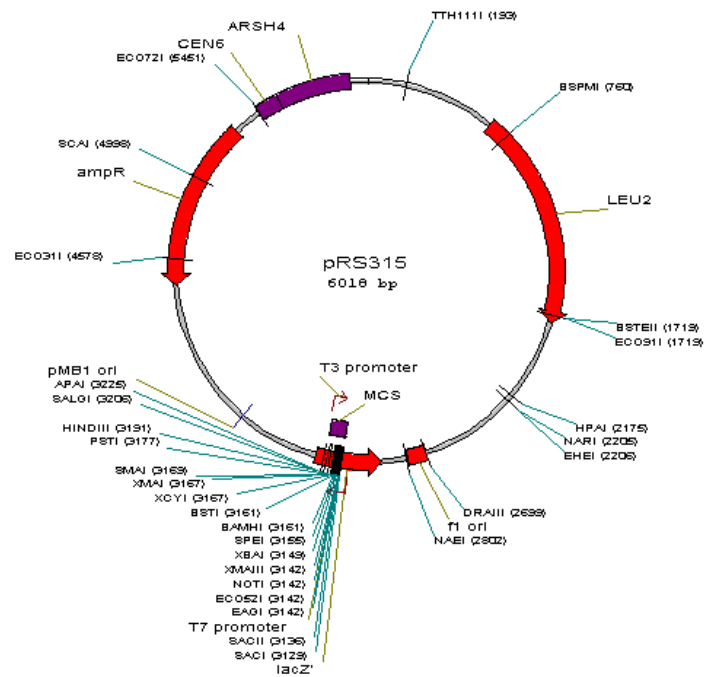**Figure II-4** (www.atcc.org): This is the pRS315 plasmid vector, referred to as the p19 vector in our lab. This figure shows the *LEU2* gene and ampicillin resistance gene, ampR, which are the selection markers for this plasmid. It also shows all the restriction digest sites on the vector. The YEp351 library utilizes the same configuration with the addition of 2 to 3 yeast genes as an insert.

not carry a yeast gene. The plasmids contain the *LEU*2 gene which enables the transformed strains, whose genome is *leu2Δ*, to grow on media lacking leucine. The plasmid also contains a gene for ampicillin resistance which serves as a bacterial selection marker if the plasmid is transformed into bacteria. For both the *osh3Δ* and *erg4Δ erg5Δ* suppression screens the positive controls were a wild type strain, 430, transformed with p19 (430+p19), a super sensitive strain, 1305, transformed with p19 (1305+p19) and a resistant strain, 1325, transformed with p19 (1325+p19). The negative control for the *OSH3* screen was an *osh3Δ* strain transformed with p19 (15B9). The negative control for the *ERG4 ERG5* screen was an *erg4Δ erg5Δ* strain transformed with p19 (DDY3).

Using the controls for each screen, it then becomes necessary to select a concentration of TTG and growing temperature to use for the screen. A high concentration of TTG will not allow anything to grow while a low concentration of TTG will not have enough antifungal effect to visually separate the differences in growth rate between the different strains. Optimal growing temperature, 30˚C, will often allow the yeast to grow too quickly, making it difficult to distinguish growth rate. Often a lower temperature of 25˚C, about room temperature, is sufficient to slow growth to a manageable rate. Determining the optimal conditions is accomplished by creating SC-Leu+TTG (SC-Leu plus a concentration of TTG) plates at differing TTG concentrations and growing the controls on them at different temperatures.

SC-Leu plates are used to maintain selection for only cells containing a plasmid. Basing the range from the appropriate TTG concentration in previous experiments in the lab, it was decided to test TTG concentrations from .02% to .05% first at intervals of .01% and then at .005%. The most promising concentrations were then tested at both 25˚C and 30˚C to determine the optimal conditions in which to conduct the screen.

The next step is to transform (**high-efficiency transformation of yeast**) the deletant strains with the YEp351 2μ library. The 2μ library is made up of the basic Yep351 high copy plasmid, but with the addition of an average of two to three yeast genes on an insert in the polylinker region of the plasmid (Figure II-4). Each transformed yeast cell should each have just one plasmid, but after replication will carry between 20 and 200 copies of this single plasmid. When the transformed strains are grown on SC-Leu media, both the *LEU2* gene and the genes contained in the insert are transcribed in high numbers.

Once transformed, the strains are then patched onto SC-Leu plates, approximately fifty to a plate. The four controls are also patched onto each plate to provide a guide for judging the growth on each individual plate, as growth may vary slightly from plate to plate. The transformants are then grown under optimal conditions at 30˚C for 36 to 48 hours. These patches are then replica plated onto SC-Leu+TTG plates, at the concentration of TTG earlier determined. This is accomplished by placing sterile velvet over a

block, and then pressing the original SC-Leu plate on the velvet, transferring yeast to the velvet. The SC-Leu+TTG plate is then pressed against the velvet, transferring yeast from the velvet onto the fresh plate. This creates an exact replica of the original on the TTG+ plate. The TTG+ plates are then stored at the temperature earlier determined and observed for growth. For a complete screen, to ensure that every gene in the yeast genome has been screened at least once, it is necessary to patch approximately 5000 transformants, which translates to over 10,000 genes screened and greater than 1.667x genome coverage.

If a transformant is observed to grow in excess of the negative control, it is termed a suppressor candidate. The candidate must be retested to ensure that the genes on the plasmid are responsible for the suppression of the super sensitive phenotype. The rate at which the yeast genome mutates spontaneously, not to mention due to the various stresses the cell is put under during transformation etc, means a significant portion of suppressor candidates do not retest positively, as the suppressed phenotype is likely due to various mutations and not a gene on the high copy plasmid.

To retest the candidate suppressor, the plasmid must be removed from the yeast. This is accomplished using the **yeast genomic miniprep** protocol. This genomic DNA will be mixed in with the plasmid, but for our purposes it is unnecessary to purify the plasmid from the rest of the DNA. The genomic DNA solution containing the plasmid is used to transform *E. coli*

(**transformation of *E. coli***). The bacteria are plated and grown on LB+Amp (Luria-Bertani media plus ampicillin) plates that select only the bacteria transformed with the plasmid. This selection is due to the ampicillin resistance gene contained in the plasmid, earlier mentioned as the bacterial selection marker. The bacteria serve to amplify the suppressor candidate plasmid to sufficient numbers to be transformed back into the yeast strain to be retested against TTG. The plasmid is obtained from the bacteria and purified using the **Qiagen Plasmid Miniprep** protocol. The purified plasmid is then transformed (**transformation of plasmid into yeast**) back into the yeast strain of interest. The re-transformed yeast strain is then patched on SC-Leu plates and replica plated onto SC-Leu+TTG plates in the same fashion as previously described. If the transformant is observed to grow better than the negative control again, it is considered a genuine suppressor.

The next step is to identify the gene that has caused the suppression of the mutant phenotype. The plasmid purified from the bacteria **Qiagen Plasmid Miniprep** protocol is sufficiently pure to be used for DNA sequencing (**DNA Sequencing**).

Mating Type Determination:

.25ml of the haploid strain in question is mixed with .25ml of a strain of known mating type and 1ml of YPAD (Yeast extract-Peptone-Dextrose medium plus Adenine) liquid media in a culture tube. Two such culture

tubes are made, one utilizing a known strain of mating type "a" and the other

with a strain of mating type "ἀ." The tubes are placed on a roller and kept at

30˚C for four hours. After the incubation period, the tubes are checked for

aggregates of cells. Aggregates of cells indicate that the cells of the strain in

question are agglutinating with and mating to the strain of known mating

type. This indicates the strain in question is of the opposite mating type, i.e.

cell aggregates in the tube containing the known strain of mating type "a"

indicate the strain in question is mating type "ἀ."

Yeast Genomic Miniprep:

To collect genomic DNA from yeast, a culture is grown overnight in

selective liquid media, usually SC-Leu (lacking leucine). The yeast are

pelleted and spheroplasted, which is the digestion of the cell wall, at 37˚C for

one hour in SEM solution. SEM solution contains Sorbitol, $NaPO_4$,

ethylenediaminetetraacetic acid (EDTA), betamercaptoethanol and

zymolyase. DTAB lysis buffer is then added and the suspension heated at

65˚C for ten minutes. The DTAB lysis buffer contains

dodecyltrimethylammonium bromide (DTAB), NaCl, Tris HCl, and EDTA.

The DNA is then extracted from the solution with chloroform. The solution

is centrifuged and the aqueous phase is collected. To the aqueous phase is

added CTAB solution containing cetyl trimethylammonium bromide (CTAB)

and NaCl. This precipitates the DNA, which is then pelleted. The pellet is

then resuspended in a 1.2M NaCl solution, precipitated again and

resuspended in TE buffer.

PCR Reaction:

   PCR, or polymerase chain reaction, is used for amplifying samples of

DNA (Figure II-5). It allows for the replication of DNA without the use of a

living organism and is highly specific based on the primers used. The PCR

solution is as follows: 1μl primer 1, 1μl primer 2, 1μl dNTPs (Deoxyribose

Nucleotide Tri-Phosphates) at 5mM each, 3μl 10x PCR buffer with $MgCl_2$,

1.5μl genomic DNA solution, 1μl Taq polymerase and double distilled water

to a total solution volume of 30μl. The solution is placed in the automatic

thermal cycling machine with the following sequence:

Step 1: 4 min at 95˚C   Step 5: Repeat steps 2-4 32x's

Step 2: 1 min at 94˚C   Step 6: 5 min at 72˚C

Step 3: 2 min at 54˚C   Step 7: up to 24 hours at 4˚C

Step 4: 2 min at 72˚C

Agarose Gel Electrophoresis:

   Gel Electrophoresis is run for several reasons. It can be run to

determine DNA size, DNA concentration, or test the result of a PCR reaction.

 For DNA electrophoresis, a .8% gel is made by heating agarose powder and

electrophoresis buffer at the correct concentration. Ethidium bromide is then
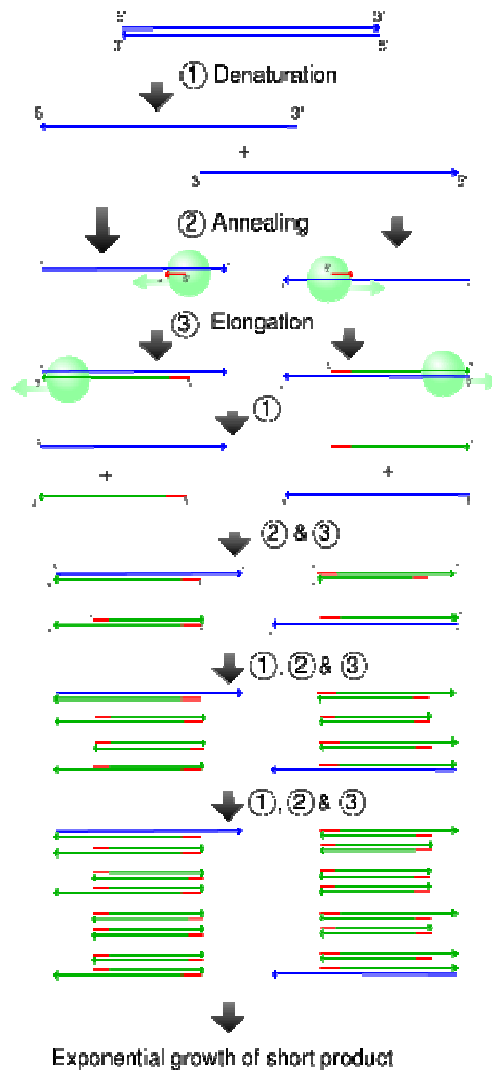
Figure II-5 (http://en.wikipedia.org/wiki/Image:PCR.svg): This figure

shows the process of DNA amplification during PCR. Two primers are used,

one for each direction. In the experiments I have performed, the primers

were ERG4ck and KanMX or ERG5ck and KanMX.

added to a concentration of about .5μg/ml and the gel is poured into a mold

with a comb that creates wells. After the gel has solidified the gel is placed

in an electrophoresis box and covered with tris-acetate-EDTA (TAE) buffer,

where the comb is removed.   The DNA, often a PCR product or product of

genomic miniprep, is mixed with a loading buffer that contains glycerol to

ensure the DNA sinks in the well, and a tracking dye. This is loaded into the

wells and an electric current is passed through the buffer and gel for ninety

minutes to two hours. This will give good separation the DNA based on size,

with the smaller fragments traveling the farthest on the gel. As the DNA

migrates towards the positive electrode, it binds ethidium bromide and

becomes visible under UV light. When determining size, a mass ladder,

which consists of several pre-cut DNA strands of various lengths, is run in

one of the wells. When determining concentration, this same mass ladder,

with differing, known concentrations of the different size strands, is run in

one of the wells and will fluoresce at different intensities based on the

concentration of DNA at that size. The fluorescence of the unknown

concentration of DNA can be compared to the fluorescence of the known

concentrations in the mass ladder. This method can also be used to purify

DNA of a particular size from other DNA fragments. If this is the goal, a low

melting temperature agarose is used, but the protocol is the same. Once the

gel has been run, the DNA band can be excised using a razor blade, the gel

melted at a low enough temperature so as not to destroy the DNA sample, and the DNA purified from the agarose.

High-efficiency Transformation of Yeast

This method of transformation is used to transform strains with the YEp351 library. The higher efficiency method is preferred because the plasmids in this library are different from one another, and thus it is necessary to transform as many cells as possible.

The strain to be transformed is grown in a liquid YPAD media overnight. The cell density is then measured using a spectrophotometer and 50ml of YPAD liquid media is inoculated to a cell density of 5×10^6 cells/ml. This culture is then incubated at 30˚C on a shaker until its cell density is 2×10^7 cells/ml, which is the result of two rounds of cell division. Two rounds of division typically take about four hours under such conditions. The 50ml culture is then harvested and separated into 5 conical, centrifuge tubes.

The centrifuge tubes are then centrifuged at 3000g (2500rpm) for 5 minutes. The YPAD medium is poured off the pelleted cells and they are resuspended in sterile H_2O and centrifuged again. The water is then poured off, the cells are resuspended in 200μl of 100mM lithium acetate (LiAc) and the suspension is transferred to an eppendorf tube. The cells are pelleted and the LiAc is removed with a pipette. The cells are then resuspended to a final volume of 100μl with 100mM LiAc again. The suspension is then divided

into ten, 50μl samples in microfuge tubes. The cells are then pelleted again

and the LiAc removed.

The "transformation mix" is then added in the order listed. 240μl

PEG (polyethylene glycol), 36μl of 1M LiAc, 25μl of single-stranded carrier

DNA usually salmon sperm DNA, and 50μl of Plasmid DNA (.1-10μg) and

water. The order is important because PEG shields the cells from the

detrimental effects of the high LiAc concentration. The cells are resuspended

in this mix by gentle pipetting and the suspensions are incubated for thirty

minutes at 30˚C. Once the incubation period is over, the cells are heat

shocked by placing the suspensions in a water bath at 42˚C for twenty to

twenty five minutes. Next, the suspensions are microfuged at 7,000rpm for

15 seconds to pellet the cells, and the transformation mix is removed with a

pipette. 500μl of sterile H_2O is used to resuspend the pellet by pipetting it

gently. 200μl aliquots are then used to plate the transformed strain onto

selective plates, SC-Leu in our instance.

Qiagen Plasmid Miniprep

The purification of plasmids from *E. coli* is an important step in any

molecular biology research. It is generally important for the plasmid solution

to be free from most contaminants. In order to purify the plasmid so that it

can be retransformed into yeast strains, or the yeast gene insert sequenced

(**DNA Sequencing**) our lab uses the Qiagen plasmid miniprep kit (Qiagen Inc., Valencia, CA).

To start, a single colony of transformed *E. coli* is grown overnight in 3ml of LB+Amp selective liquid media. The bacteria are pelleted by centrifugation at 6000g for 15 minutes and the supernatant removed. The pellet is then resuspended in .3ml of buffer P1, provided in the kit. Then .3ml of buffer P2 is added to the suspension, the tube is inverted several times to mix the buffers and the suspension is incubated at room temperature for 5 minutes. .3ml of buffer P3, which has been chilled on ice, is then added and the suspension is mixed and chilled on ice for 5 minutes. The suspension is then centrifuged for 10 minutes at 14,000g, maximum speed for most microcentrifuges. While the suspension is being centrifuged, a Qiagen-20 column is equilibrated by allowing 1ml of QBT buffer to pass through it. Once the suspension is removed from the centrifuge, the supernatant is removed immediately and applied to the Qiagen-20 column and allowed to enter the resin by gravity flow. The column is then washed twice with 2ml aliquots of buffer QC. The column is then placed over an eppendorf tube and the DNA is eluted with .8ml buffer QF.

The eluted DNA is then precipitated by adding .56ml isopropanol to the solution, mixing and then centrifuging the solution at 10,000rpm for 30 minutes. The supernatant is carefully decanted and the DNA pellet is resuspended in 1ml 70% ethanol. This solution is centrifuged for 10 minutes

at 10,000rpm. The supernatant is carefully decanted again and the pellet allowed to air dry for 5 to 10 minutes. The DNA pellet is then resuspended in 50μl of TE buffer. It is then ready for quantitative gel analysis (**Agarose Gel Electrophoresis**), for transformation into yeast strains (**Transformation of Plasmid into Yeast**), or for **DNA Sequencing**.

Composition of Qiagen buffers:

P1 (Resuspension Buffer): 50mM Tris Cl, 10mM EDTA, 100μg/ml RNase A

P2 (Lysis Buffer): 200mM NaOH, 1% SDS

P3 (Neutralization Buffer): 3M Potassium Acetate (KAc)

QBT (Equilibration Buffer): 750mM NaCl, 50mM MOPS, 15% Isopropanol, .15% Triton® X-100

QC (Wash Buffer): 1M NaCl, 50mM MOPS, 15% Isopropanol

QF (Elution Buffer): 1.25M NaCl, 50mM Tris Cl, 15% Isopropanol

Transformation of *E. coli*

The transformation of *E. coli* is a common method used in genetics and biochemistry research. For our purposes, *E. coli* are used to amplify selected plasmids so they can be retransformed into yeast strains. The first step is to create competent bacterial cells. This is accomplished by first growing the *E. coli* cells overnight in LB media. 49.5ml of LB media + 10mM $MgCl_2$ is then inoculated with .5ml of this overnight culture. It is then allowed to grow for two to three hours at 37°C. The flask containing the

suspension is then cooled on ice for fifteen minutes. The culture is the spun

at 3000g (2500rpm) for five minutes. The supernatant is then discarded and

the pellet resuspended in half volume of ice cold .1M CaCl$_2$. This suspension

is put on ice for an additional thirty minutes. This suspension is divided into

centrifuge tubes and spun for five minutes at 3000g (2500rpm). This

supernatant is discarded and the pellets resuspended in 1/20 volume ice cold

.1M CaCl$_2$+ 15%glycerol. This suspension is left to sit in a 4˚C refrigerator

for 24 hours, as competency increases in 24 hours, but subsequently falls.

After 24 hours, the suspension is divided into 200μl aliquots of competent

cells, which can be frozen at -80˚C.

Starting with these pre-chilled tubes of competent cells, in order to

transform the bacteria 2μl of DNA solution obtained from yeast by the **Yeast**

Genomic Miniprep method is added. The tube is left on ice for 30 minutes

and then heat shocked for 45 seconds at 42˚C. The tube is then returned to

the ice for approximately two minutes. Then, 100μl of LB media that has

been pre-warmed to 42˚C is added to the tube. Once mixed, the culture is

plated on a selective, LB+Amp (Ampicillin) plate. The plates are then left

overnight at 37˚C.

One Step Transformation of Plasmid into Yeast

This method of transformation does not have the same level of

transformation efficiency as the high-efficiency method, but is sufficient for

the purpose of transforming with a single, purified plasmid. It is also much less complex and time consuming, making it the preferred method when high transformation efficiency is not necessary. To transform the yeast, a culture of the yeast strain to be transformed is grown overnight in selective media, usually SC-Leu. The cells are transferred to an eppendorf tube and pelleted. The supernatant is then removed. The cells are then resuspended in one step buffer, which contains lithium acetate (LiAc), polyethylene glycol (PEG), and β-mercaptoethanol. A single stranded DNA carrier, like salmon sperm DNA, is then added along with the plasmid. After mixing, the tube is placed in a 45˚C water bath for thirty minutes. After this incubation period, the cells are pelleted again and the supernatant is removed. The cells are then resuspended in SC-Leu liquid media and then plated on SC-Leu plates, in order to select only the transformed cells for growth.

DNA Sequencing

The DNA sequencing service we use is performed using BIG DYE™ Version 3 terminators from Applied Biosystems and are run on an ABI 377 DNA Sequencer at SUNY Upstate Medical Center's DNA core facility. The method is known as automated fluorescent sequencing and is run in a similar fashion to PCR reactions, with some slight differences. The reaction is run in a thermal cycler using a single primer to start a unidirectional polymerase reaction (whereas in PCR two primers are used and the polymerase reaction

goes in both directions) and also utilizes ddNTP's (di-deoxynucleotide triphosphates) that are labeled with fluorescent dyes.

These ddNTP's terminate the replication reaction at random intervals. They terminate the polymerase reaction when integrated into the new DNA strand due to the lack of an oxygen molecule on the ribose moiety to which the next nucleotide can be fixed. Due to the large amount of DNA present, this should terminate a detectable number of DNA strands at each base in the sequence. The last nucleotide in the sequence, the ddNTP, is labeled with a fluorescent dye, ddATP appears green, ddTTP appears yellow, ddCTP appears red and ddGTP appears blue.

After the DNA is purified, the strands can be run on a gel and read by an automatic sequencing machine which can read the fluorescent dyes, and which will display the DNA sequence. This is only a partial sequence, however, as the reaction will only accurately replicate 500-700 base pairs. The sequence is then used to run a BLAST search on the yeast genome database website, www.yeastgenome.org, which will show us what part of the yeast genome corresponds to the insert in the 2μ library for the suppressor we have seqeunced. Once this information is acquired, another primer can be selected to sequence the opposite side of the insert. Once this sequence is obtained, a BLAST search of both the sequences will reveal the two ends of the insert and what yeast genes lie in between.

The first step in the sequencing reaction procedure is to prepare the reaction mixture. It contains: 8μl "Terminator Reaction Ready Mix," 200-500ng double stranded template DNA, 3.2pmol primer, and deionized H_2O to a total volume of 20μl. The "Terminator Reaction Ready Mix" contains AmpliTaq® DNA polymerase, dNTP's, and BIG DYE™ v3.0 fluorescently labeled ddNTP terminators. The mixture is then mixed well and spun briefly before being overlaid with 40μl of mineral oil. The tubes are then placed in the thermal cycler with the following steps repeated 25 times:

1: Rapid thermal ramp* to 96˚C   4: 50˚C for 5 seconds

2: 96˚C for 10 seconds   5: Rapid thermal ramp to 60˚C

3: Rapid thermal ramp to 50˚C   6: 60˚C for 4 minutes

After the 25 cycles: Rapid thermal ramp to 4˚C, hold until ready to purify

*Rapid thermal ramp corresponds to 1˚C/second

The contents of the tube are then spun down for purification. Purification is necessary because excess dye terminators will interfere will the reading of the shortest strands and could interfere with base counting as well. The first step in ethanol purification is to remove the reaction mixture from under the oil and place it in a 1.5ml microfuge tube. To the microfuge tube is added 16μl deionized water and 64μl 95% ethanol and mixed well. The tubes are then left at room temperature for at least 15 minutes and preferably longer to precipitate the extension products. The tubes are then spun in a microcentrifuge at maximum speed. The supernatant is then

aspirated carefully and completely, as unincorporated dye terminators are in the supernatant. 250μl of 70% ethanol is then added and the pellet resuspended by careful vortexing. The tubes are then spun for 10 minutes at maximum speed. The supernatant is then aspirated carefully again and the pellets allowed to dry.

The pellets are then prepared to be run on a 4.5% acrylamide gel and analyzed using the ABI 377 DNA Sequencer. Each pellet is resuspended in 6μl of a loading buffer which consists of a 5:1 ratio of deionized formamide and 25mM EDTA with 50mg/ml dexetran blue. The samples are then vortexed gently and spun briefly. The samples are heated to 95˚C for 2 minutes to denature any DNA that had bound, and 2μl of each sample placed in a separate well. The gel is then set up to run in the automated sequence analyzer and the machine does the rest.

Results:

Creation of *erg4Δ erg5Δ* Double Mutants:

In attempting to create the *erg4Δ erg5Δ* double mutants

approximately 45 tetrads were dissected. Of these, 16 grew in a "2 and 2"

pattern when replica plated onto YPAD+KAN media. This is 35.5% of the

tetrads dissected. This number is not quite as high as would be expected, i.e.

50%, perhaps due to chance, but also possibly due to human error. For

example, some of the "tetrads" that were dissected were not really tetrads, but

clumps of four cells that were mistaken to be tetrads when looked at through

the microscope. This resulted in 32 (16 x 2) double mutant candidates.

Of these 32 candidates, eight grew on SC-Lys but not on SC-Met

media. This is performed because the strains we have decided to use are

met15Δ. Of these eight candidates, four were mating type "a." Remembering

that we have decided to use mating type "a" cells and that all of the labs

mating type "a" cells are *met15Δ*, the four that are *met15Δ* and mating type

"a" are the usable double mutant candidates. Genomic DNA was obtained

from the four usable candidates and two PCR reactions run on each

candidate's DNA. The first used the ERG4ck and KanMX primers. The

second used the ERG5ck and KanMX primers (Figure III-1). Gels were then

run to allow me to determine which candidates were in fact actual double

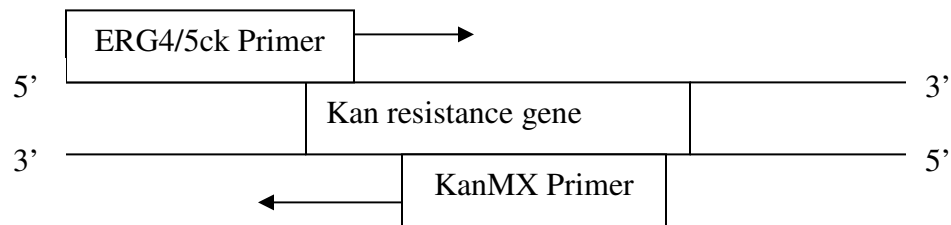mutants. The first gel was run using the samples from the ERG4ck and

Figure III-1: This figure depicts how the primers anneal during the PCR reaction. If the *ERG4* or *ERG5* gene has not been replaced by the Kan resistance gene, then only the ERG4 or ERG5 primer will have a binding site and no band will be present on the gel. If the genes have been replaced, only then will the KanMX primer have a binding site and the PCR reaction successfully take place, resulting in a band on the gel.
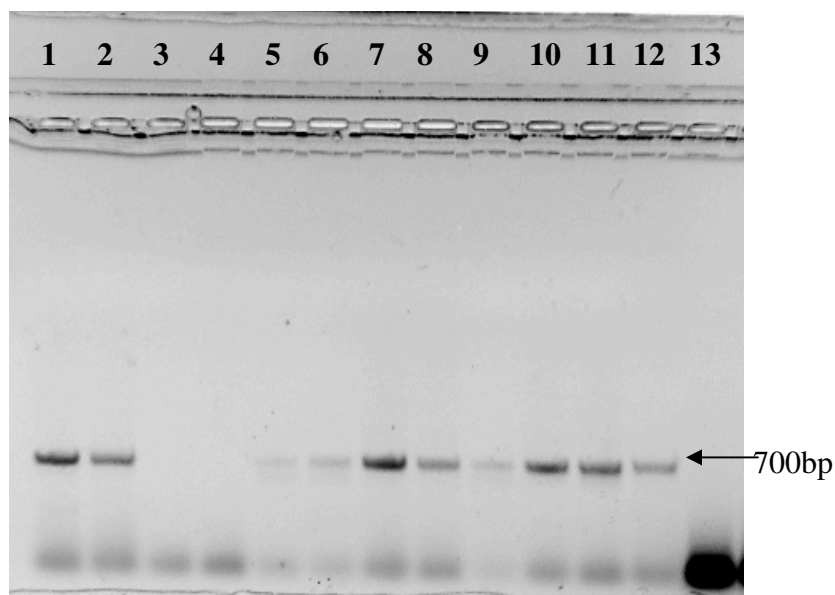
Figure III-2: This gel was run on the four double mutant candidates and two single mutant strains. All the lanes in this gel depict PCR reactions run with the ERG4ck and KanMX primers. Lanes 1 and 2 show the *erg4Δ* genomic DNA reaction, lanes 3 and 4 show the *erg5Δ* genomic DNA reaction, the four double mutant candidates, C1-C4, were run on the next eight lanes, two lanes to each candidate. Lane 13 is a mass ladder that, for some reason, did not work. Though it doesn't print clearly, the gel shows bands in all lanes except lanes 3 and 4. This result is as expected, as the *erg5Δ* should not show a product with the ERG4ck primer, and shows all the double mutant candidates to be *erg4Δ*.
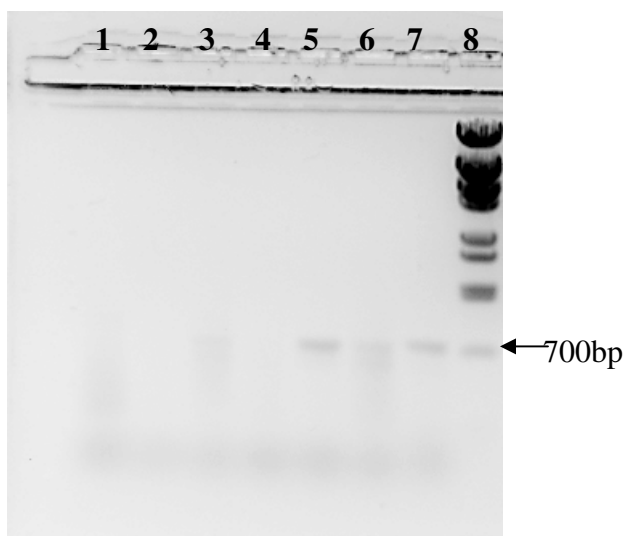
Figure III-3: This gel was also run on the 4 double mutant candidates as well as the 2 single mutants, but utilizing the ERG5ck and KanMX primers. Lane 1 is the *erg4Δ* single mutant strain and lane 2 is the *erg5Δ* single mutant strain. Lanes 3-6 are the double mutant candidates, C1-C4. Lane 7 is a sample run from another experiment and not pertinent to the identification of the double mutants. Lane 8 is a mass ladder. The gel shows that bands appeared at 700bp in lanes 3, 5 and 6. A band did not appear in lanes 2 and 4 which was unexpected. Lane 2 was rerun in the next gel (Figure III-4) because it should have had a band. The gel indicates that the double mutant candidate in lane 4, C2, was not *erg5Δ*.
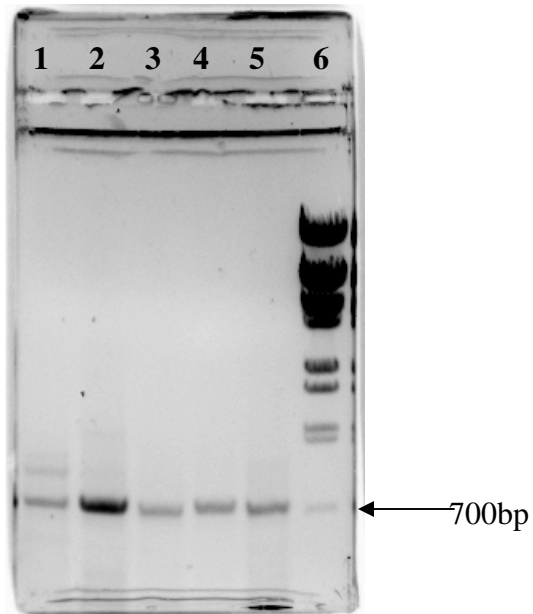
Figure III-4: This gel was run with two of the candidates that showed the strongest bands in the previous gels and intended to prove conclusively they are double mutants. Lane 1 is the retest of the *erg5Δ* single mutant with the ERG5ck and KanMX primers. Lane 2 is C4 with the ERG4ck and KanMX primers and lane 3 is C4 with the ERG5ck and KanMX primers. Lane 4 is C3 with the ERG4ck and KanMX primers and Lane 5 is C3 with the ERG5ck and KanMX primers. Lane 6 is a mass ladder. A band appears in all lanes at 700bp, which shows that the *erg5Δ* strain is really an *erg5Δ* strain and that both C3 and C4 are truly double mutants.

KanMX PCR reaction (Figure III-2). The second gel was run using the

samples from the ERG5ck and KanMX PCR reaction (Figure III-3). From

these gels, three of the four samples were shown to be genuine *erg4Δ erg5Δ*

double mutants. To be absolutely sure, the two PCR reactions were run again

on the two samples with the strongest bands in the first two gels, and the

resultant PCR samples run on a gel (Figure III-4). This gel confirmed the

two candidates are in fact genuine double mutants. These two *erg4Δ erg5Δ*

double mutants were transformed with the p19 plasmid to create control

strains, named DDY3 and DDY4. Additionally, an *erg4Δ* single mutant

haploid was transformed with the p19 plasmid to create the DDY1 control

strain and an *erg5Δ* single mutant likewise transformed to create the DDY2

control strain.

Selection of TTG concentration for suppressor screening:

For the *erg4Δ erg5Δ* double mutant screen the wild type (430), super-

sensitive (1305), resistant (1325) and *ERG4Δ ERG5Δ* double mutant (DDY3)

strains were grown on SC-Leu plates with TTG concentrations of .02%,

.03%, .04% and .05% at 30˚C overnight. It was determined that .02% and

.03% were the most promising concentrations, as .04% and .05% did not

allow sufficient growth even in the resistant controls. SC-Leu+ .02%, .025%

and .03% were then inoculated with the control strains and grown at both

30˚C and 25˚C. It was determined that .025% TTG concentration at 25˚C

provided the best separation of growth patterns and was therefore the most conducive to identifying suppressors. The same process was repeated with the *osh3Δ* control strains with the same results. Both screens were therefore conducted on SC-Leu+.025%TTG at 25˚C.

High Copy Suppression Screen:

For the *erg4Δ erg5Δ* double mutant screen 30 plates were patched with a total of 1,322 patches, each representing a different transformant. Each transformant screened represents one plasmid screened and each plasmid contains two to three genes. Thus, the 1,322 patches correspond to approximately 1322 x 2.5= 3,300 genes, or more than half of the predicted 5,800 genes comprising the yeast genome. However, no suppressor candidates were identified after such a substantial number of genes were screened, so the project was abandoned.

For the *osh3Δ* screen, 17 plates have been patched thus far with a total of 909 patches. This corresponds to approximately 909 x 2.5 = 2,270 genes tested, about 40% of the yeast genome. Of these patches, six were identified as suppressor candidates. Candidates 1, 3, 4, 5 and 6 grew in excess of the negative control, similar to the wild type control. Candidate 2 grew well in excess of the wild type control and even in excess of the resistant control. The six candidates represent 0.66% of the plasmids screened. Of the six suppressor candidates, four candidates, 2, 3, 5 and 6, retested as genuine

suppressors. These four candidates represent 0.44% of the plasmids screened

(Table III-1).

Mutant Strain	Transformants Screened	Suppressor Candidates	%	Genuine Suppressors	%
erg4Δ erg5Δ	1322	0	0	0	0
osh3Δ	909	6	0.66	4	0.44

Table III-1: This table summarizes the results obtained from the two high

copy suppression screens. The candidate suppressors are in the process of

being sequenced at the time of the publication of this Capstone Project.

Discussion:

The distribution of lipids among the cell's various organelles is essential for the proper functioning of the cell. The processes by which cells regulate the distribution of lipid particles like phospholipids, glycolipids, sphingolipids, proteins and sterols, are poorly understood. Because improper sterol regulation and trafficking can lead to both high and low cholesterol levels in the human circulatory system, and improper cholesterol levels have been associated with a range of diseases from heart disease to Alzheimer's disease, understanding the mechanisms by which cells regulate membrane composition of sterols is essential.

Yeast, as a simple eukaryote, is an essential model system for discovering the methods by which cells regulate their membrane compositions. The members of the Erdman lab, myself included, are utilizing high copy suppression screens in order to understand membrane homeostasis. I have begun high copy suppression screens on the *osh3Δ* and *erg4Δ erg5Δ* double mutant strains. The *erg4Δ erg5Δ* double mutant screen did not yield any suppressors after a significant number of genes had been screened, and the screen was thus abandoned. In the *osh3Δ* screen six candidate suppressors were identified, four of which retested positively as genuine suppressors. The four suppressors of the *osh3Δ* phenotype need to be sequenced and the genes responsible for the suppression identified.

Thus far, we have learned something is not right with the *erg4Δ* *erg5Δ* double mutant screen. The double mutant is viable, but it is unclear why the screen did not yield any positive results after so many genes were screened. It is possible no suppressors could overcome the deletion of both genes, so screens on the *erg4Δ* and *erg5Δ* single deletion strains is a possibility for future research in the Erdman lab. What we have learned from the *osh3Δ* screen is unclear until the genes responsible for the suppression can be identified. It is hoped that these genes, which may have a similar or overlapping function with the *OSH3* gene, will give clues to the function of Osh3p.

The future direction of the project is to continue the *osh3Δ* screen. At this point, only about 1/5 of the transformants that are necessary to ensure complete genome coverage have been screened. In addition, the suppressors already identified, and any resulting from the continuation of the screen, will be sequenced and their identities discovered. Deletion mapping and subcloning strategies will be used to map the genes responsible for the high copy suppression within the genomic DNA inserts of the different library plasmids will be mapped to single genes (the plasmids typically contain between one and as many as five open reading frames depending on ORF sizes and insert size). Also, individual screens may be carried out on the *erg4Δ* and *erg5Δ* single mutant strains, instead of the failed double mutant screen. Once these screens are completed, future work will depend heavily

on the identity and nature of the suppressors. If any suppressors have a particularly interesting function, an unknown function or their function is involved in membrane homeostasis or composition, these are likely candidates for future research in the Erdman lab. It is also possible that any of the OSH family of genes was responsible for the suppression, including *OSH3* itself. If this is the case, it is not likely these genes would be pursued any further. Discovering the nature of the suppression of the genes selected for further research is the likely first step in this future research.

One of the first experiments will be to determine whether the suppressor gene will increase the resistance of wild type strains to TTG. If the suppressors do increase resistance of the 430 strain to TTG, it would indicate the over expression of the suppressor gene increases resistance in general, and is not specific to the deleted gene. If the suppression is shown to be specific to the deleted gene, future experiments would seek to identify the exact nature of the interaction, which may include two-hybrid screens or protein co-purification experiments among other possibilities, depending on the functions of the suppressor genes.

Though much of my time was tied up in a screen that had no results, and the other screen is far from complete, I hope to have laid the groundwork for future research on these genes and the pathways associated with membrane sterol composition. I also hope that this future research may find

broad application beyond mere knowledge of the cell and be applied to

medical and pharmaceutical fields.

Sources Cited

Beh, Christopher T., and Jasper Rine. "A Role for Yeast Oxysterol-Binding
     Protein Homologs in Endocytosis and in the Maintenance of Intracellular
     Sterol-Lipid Distribution." <u>Journal of Cell Science</u> 117 (2004): 2983-96.

Beh, Christopher T., Laurence Cool, John Phillips, and Jasper Rine.
     "Overlapping Functions of the Yeast Oxysterol-Binding Protein
     Homologues." <u>Genetics</u> 157 (2001): 1117-40.

Goffeau, A, B G. Barrell, H Bussey, and R W. Davis. "Life with 6,000
     Genes." <u>Science</u> 274.5287 (1996): 546, 563-7.

Im, Young Jun, Sumana Raychaudhuri, William Prinz, and James Hurley.
     "Structural Mechanism for Sterol Sensing and Transport by OSBP-
     Related Proteins." <u>Nature</u> 437.7055 (2005): 154-58.

Morrissey, John, and Anne Osbourn. "Fungal Resistance to Plant Antibiotics
     as Mechanism of Pathogenesis." <u>Microbiology and Molecular Biology
     Reviews</u> 63.3 (1999): 708-24.

Raychaudhuri, Sumana , Young Jun Im, James H. Hurley, and William A.
     Prinz. "Nonvesicular Sterol Movement from Plasma Membrane to ER
     Requires Oxysterol-Binding Protein-Related Proteins and
     Phosphoinositides." <u>The Journal of Cell Biology</u> 173.1 (2006): 107-19.

27, February 2007 <http://en.wikipedia.org/wiki/Image:PCR.svg>

27, February 2007 <www.phys.ksu.edu/gene/a2f3.html>

3, March 2007 <http://syllabus.syr.edu/bio.seerdman/TTG>

15, April 2007 <www.doctorfungus.org>

15, April 2007 <www.atcc.org>

15, April 2007 <www.biochemsoctrans.org>