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**Identifying Proteins that Interact with the Novel
Saccharomyces cerevisiae Proteins PDR19 and PDR20 and
Bioinformatic Characterization of Genes Involved in TTG
Cellular Responses**

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors

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Honors Capstone Project in Biochemistry

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ABSTRACT

One of the most critical structures in cellular biology is the plasma membrane, due to its ability to respond to environmental stresses. *Saccharomyces cerevisiae* is a model, single-celled eukaryote that has been used to investigate many aspects of cell biology. A recent genetic screen in yeast for plasma membrane homeostatic proteins identified three related proteins of unknown molecular function that participate in these processes. These proteins, termed PDR19, PDR20, and PDR21 for Pleiotropic Drug Resistance, are each approximately one hundred amino acids in size and share a small conserved domain, namely the core sequence KITRYDL. In the case of PDR21, the core sequence is VITRHDL. The coding sequences for this set of proteins are found in the ORFs YGR035c, YLR346c and YPR145w-a, respectively. A triple mutation of these genes led to an observed decrease in membrane homeostasis, when cells were treated with the membrane-disrupting compound digitonin, natural products that disturb membranes and in the presence of the clinical antifungal drug amphotericin B. The observed phenotype suggests this set of novel proteins functionally regulate membranes in response to membrane-altering conditions, as an observed fifty-fold increase in membrane sensitivity of the triple mutant was observed. In order to help determine the molecular function(s) of the PDR proteins, a GAL4 two-hybrid system is being used to screen for proteins that may associate with the PDR 19/20/21 family proteins and help

mediate their cellular functions. That this system can be used has been confirmed through negative autoactivation tests involving a Gal4DBD-PDR fusion construct and done in a modified Y187/Y190 mating strain carrying the pACT II activation domain plasmid containing the AD_{Gal4}. Plasmid sequencing of the Gal4DBD-PDR fusion proteins is in process to help confirm proper cloning of the bait proteins in addition to library screening. In addition to this work, bioinformatic characterization of genes involved in TTG cellular responses was conducted. In a previous screen in the Erdman lab, 4,851 deletion strains were screened, of which 991 strains demonstrated a degree of sensitivity or resistance to TTGs. In an attempt to further understand and classify these results, a bioinformatics tool was used to reveal underlying modes of genetic control governing the range of observed phenotypic sensitivities.

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INTRODUCTION

Perhaps one of the most crucial structures in cellular biology is the plasma membrane, because of its ability to respond to environmental stresses. This is particularly the case with *Saccharomyces cerevisiae*, as they are single-celled eukaryotes that naturally remain in contact with the surrounding environment. In order to regulate, and often times resist, environmental challenges such as osmotic pressure and chemical uptake, many cellular organisms adapt specialized plasma membranes, as is the case with *S. cerevisiae*. The plasma membrane classically consists of a lipid bilayer membrane with distinct regions of hydrophilicity and hydrophobicity. In addition to the presence of phospholipids, several other components, such as sterols, provide structural support and fluidity to a membrane bilayer. Moreover, several gene products regulate ion and molecular channels in yeast membranes, as well as signal transduction associated with changes in permeability. These components of the plasma membrane rarely act alone, often inducing multiple pathways. The specific mechanisms of plasma membrane structure, fluidity, and channel regulation are of interest, for most biological systems have evolutionarily adopted the plasma membrane with little variation. *S. cerevisiae* is an ideal experimental model system used in studying such underlying membrane mechanisms, due to its ease of genetic manipulation and analysis of phenotypic expression. Understanding the mechanisms and

genetic components regulating membrane permeability and homeostasis will lead to a greater understanding in related biological systems.

PLASMA MEMBRANE STRUCTURE

The outermost layer of the cellular envelope in yeast is the cell wall. Essentially, this structure provides support and rigidity to the entire cell. Beneath the cell wall is the plasma membrane, which is responsible for providing a semi-permeable barrier for hydrophobic molecules greater than 600 Da^[7]. The plasma membrane is composed of several different types of lipids, which possess different chemical and physical properties. This finding suggests a possible mechanism by which proteins within the membrane associate with distinct lipids^[7]. One of the largest classes of lipids present in the membrane by percentage is the sphingolipids. They may contribute as much as thirty percent of the total phospholipid content present in the plasma membrane^[7]. The fatty acid chains present in the plasma membrane of *S. cerevisiae* largely include oleic acid (18:1) and palmitoleic acid (16:1)^[7]. Essentially the packing level of these fatty acyl chains is what contributes to the overall fluidity of the plasma membrane. Tight packing of the fatty acyl chains results from increases in acyl chain length and consequently favors a membrane that is relatively more rigid, disallowing extensive lateral movement or permeation by hydrophobic solutes. Equally important for the physical attraction of proteins, the head

groups present on the lipids influence membrane proteins, as well as the overall electrical potential of the membrane.

While the membrane largely consists of a phospholipid bilayer, several integral proteins and sterol molecules play a key role in transporting solute molecules across the membrane. Through vesicular transport, proteins are produced on rough endoplasmic reticulum and travel to the plasma membrane. Transport vesicles bud off of the ER and fuse with the Golgi apparatus, initiated by a Ras-type GTP binding protein^[7]. Through a GDP-GTP exchange protein the SAR1 protein becomes active, thereby initiating vesicular budding from the ER. The GTP is consequently hydrolyzed upon completion of the formation of the vesicle^[7]. Like most cellular processes, vesicular trafficking and membrane construction is the result of several interacting genetic pathways and gene products. For instance, a single mutation in the GTP-binding protein, Ypt1p, results in the consequential buildup of membranes within the cell^[7]. In mammalian cells, the vesicular fusion process is similar, but involves three complexes known as SNAPs, v-SNARE, and the target fusion membrane, t-SNARE^[7].

Several key proteins remain in contact with the plasma membrane. These proteins allow for ATP binding, diffusion, and other types of transport. One of the larger families of proteins present in the membrane is the ABC transporter proteins. Such proteins contain a conserved ATP-binding cassette domain that allows these protein transporters on the

membrane to bind with ATP^[7]. The protein composition of a plasma membrane, along with phospholipid and sterol content, greatly determines the degree of permeability allowed by the membrane. In the most simple of cases, small solutes passively diffuse across the membrane without an expenditure of ATP. Not surprisingly, most small solutes are more hydrophilic in chemical composition than hydrophobic. A more specialized method of passive diffusion involves the use of channels that span the width of the bilayer. Like most eukaryotes, yeast membranes include a potassium, K^+ , channel that closely regulates potassium efflux. The next prevalent method of solute transport in yeast cells involves the active establishment of an electrochemical gradient, known as secondary transport. The proteins involved in secondary transport are thereby responsible for establishing and maintaining a homeostatic gradient at all times. In uniport secondary transport, the gradient is created by the passing solute and only transports one type of solute. In yeast cells, this is often observed with monosaccharide transport. In a symport system, the carrier protein co-transport two molecules in the same direction. Ion secondary transport often occurs concurrently with the influx of sodium ions across the membrane. Likewise, symport proteins regulate the transport of larger disaccharide molecules. Lastly, in antiport transport, two solutes are simultaneously transported in opposite directions. Typically, this maintains the electrochemical potential within and outside of the cell. A classic example of this particular type of transport is found in

most eukaryotic cells, in the form of an evolutionarily conserved sodium-proton pump. With the influx of sodium cations, there is an efflux of protons^[7]. Understanding these modes of protein-mediated transport provides critical insight in studying membrane homeostasis.

In almost all plasma membranes, the sterols play an integral role in membrane homeostasis and overall permeability. Geometrically, sterols interrupt lipid interactions and layering within the membrane, thereby contributing both a degree of asymmetry and plausibly influencing integral protein activity^[7]. In yeast, the ergosterol biosynthesis pathway largely directs sterol integration in the plasma membrane. Ergosterol is analogous to the mammalian sterol, cholesterol. Like most sterols, ergosterol is formed in the ER, and is synthesized from mevalonic acid^[7]. This sterol is of particular interest since many naturally occurring environmental toxins complex with ergosterol, consequently disrupting the plasma membrane bilayer. Interestingly, the gene products found within the ergosterol biosynthesis pathway are currently targets of interest in antifungal research. Perhaps one of the most effective approaches in antifungal technology seeks to deplete the plasma membrane of its structural integrity and selective permeability. Sterol binding by a natural or clinical drug, currently azole-based, may cause disruption of the plasma membrane. Understanding the underlying genetic mechanisms of membrane homeostasis thus proves not only to be of genetic value, but,

perhaps more importantly, holds great clinical value in developing more effective antifungals.

THE YEAST PDR NETWORK

My research project attempts to understand some of the underlying genetic mechanisms of plasma membrane resistance to naturally occurring antifungal compounds. Perhaps one of the largest genetic contributors to observed phenotypic membrane resistance, in yeast, is the pleiotropic drug resistance gene network. The PDR network contains a family of genes that participate in cellular activities, such as tolerance to membrane disturbing compounds and functioning in membrane transport^[1]. Interestingly, several transcription regulators control the expression of genes functioning as membrane efflux pumps. Such pumps have been shown to provide a mechanism of drug resistance. Consider the transcriptional regulators PDR1, PDR3, PDR7, and PDR9. Together, they control the gene expression of PDR5, whose encoded protein belongs to the ABC protein family and functions as a drug efflux pump^[1]. PDR1 and homolog, PDR3, regulate YOR1, SNQ2, and STE6. Snq2p is analogous to the Pdr5p efflux pump and most likely functions in the cytosol. Together, these proteins are responsible for observed resistance to various toxic compounds such as cycloheximide, triterpene glycosides (TTGs), and antimycin^[7]. Not surprisingly, many PDR proteins are capable of transporting compounds through their ATPase activities. Additionally, the YAP regulatory stress response network contributes to

genetically controlled drug resistance^[7]. It is believed that both the PDR and YAP networks function closely together to establish drug resistance.

Mechanistically, many antifungal drugs target the electron transport chain of the mitochondria, consequently disrupting ATP production and thereby eliminating membrane homeostatic processes. Interestingly, though, in cases of phenotypic resistance to antifungals, signals from distressed mitochondria activate the PDR network. Specifically, PDR1 and PDR3 transcription factors undergo substitution mutations and results in an upregulation of PDR5^[3], which consequently leads to an overexpression of membrane efflux pumps. Moreover, both the loss of mitochondrial genes and the inner mitochondrial membrane protein, Oxa1p, results in the upregulation of PDR3^[1]. Furthering these findings, in 2000 it was observed that phenotypic resistance to the clinical antifungal, mucidin, was the result of a mutation in the PDR3 gene^[4]. When compared to wildtype strains, deletion strains for the PDR1 and PDR3 genes demonstrated the highest degree of drug sensitivity^[4], suggesting these homologs are critical in pleiotropic drug resistance. Furthermore, when a wildtype strain was grown in the presence of mucidin, there was almost a three-fold increase in the concentration of the Pdr5p efflux pump protein when compared to control cells^[4].

In general, it is believed that the PDR network is responsible for both efflux pumps and lipid membrane trafficking specific to drug resistance. When considering the effects of both deleting the PDR1 and

PDR3 genes and the normal homeostatic processes involved with the plasma membrane, it becomes apparent that disturbing either the efflux pumps or ATP binding cassette proteins will lead to phenotypically observed sensitivity. Moreover, given that the PDR network and related genetic pathways are numerous, a disturbance in any one particular target gene transcript could disturb drug resistance. Moreover, it is likely that some of the current uncharacterized targets of the PDR network specifically contribute to either the efflux pump, or are involved in lipid membrane trafficking and/or cytosolic membrane ATP activity.

IDENTIFYING THREE PDR PROTEINS of UNKNOWN MOLECULAR FUNCTION

In an attempt to further understand the underlying genetic mechanisms of pleiotropic drug resistance, a deletion screen in the Erdman lab (Syracuse University) attempted to understand phenotypic resistance to glycosides of triterpene C30 compounds. These compounds are saponins that occur widely in plants as defense mechanisms. Amongst these naturally occurring defense compounds are chaconine, glycyrrhizic acid, gummosogenin, machaeric acid, and machaerinic acid. These compounds disturb plasma membranes in a variety of ways, including association with ergosterol molecules within the bilayer. Analogous to nystatin, the plasma membrane ATPase and chitin synthase activity are often pathway antifungal targets in sterol-rich domains.

Interestingly, it has been suggested that sterol-rich regions contain chitin synthases, while sterol-poor domains contain ATPases^[7]. In either case, it becomes evident that drugs complexing with sterol bilayer molecules disturb normal membrane homeostatic processes.

A high copy suppression screen was carried out in an attempt to identify and isolate gene interactions with SIP3, whose protein resides in the plasma membrane. SIP3 is known to positively associate with SNF1, a protein kinase involved with both nuclear histone phosphorylation and glucose repression states^[2]. The SIP3 gene ontology (GO) molecular function classifies the gene for its involvement in transcription cofactor activity and is specifically responsible for the positive regulation of transcription from the RNA polymerase II promoter^[2]. A high copy suppression screen involves the overexpression of candidate genes, and in this case positively identifies genes that influence resistance to TTG. In this particular screen, the SIP3 strain exhibits super sensitivity to TTG. In an experiment conducted by Gary Franke of the Erdman lab, 5,200 transformant colonies and high copy plasmids were screened by transforming a yeast 2 μ library into a SIP3 strain. Of the 5,200 transformant colonies screened for TTG sensitivity, 31 high-copy suppressors were isolated. 17 of these were retested and shown to cause TTG resistance. Of these 17, a total of 11 unique high-copy suppressors were isolated. With an average of 2-3 genes per insertion, and a yeast genome spanning approximately 6,000 genes, an estimated 11,700

genes, or nearly 2x the genome, were screened for TTG resistance (ttgR) phenotypes.

Of the candidate high-copy suppressors of the sip3 Δ strain, three genes of unknown molecular function were identified with coding sequences found in the following ORF's: YGR035c and YPR145c-a. Among other suppressor genes identified, PDR16 and LAG1 were isolated, both regulated by the PDR network. Of the total candidate suppressor genes, most fell under three general molecular functions, namely pleiotropic drug resistance, vesicular trafficking, and stress response. A further investigation and understanding of the PDR network suggests that YGR035c and YLR346c are direct target genes of unknown molecular function regulated by PDR8, YRR1, and/or YRM1. It is likely that TTG resistance may be caused by an overproduction of pleiotropic drug resistance proteins. For the most part, PDR1/3, YRR1, and YAP1 all contribute to drug resistance. Most of the PDR genes encode either ABC transporters, MFS permeases, or are involved in membrane metabolism. YGR035c, YLR346c, and YPR145c-a are regulated by the PDR network due to their observed phenotypic behaviors in response to membrane disturbing compounds. YGR035c, YLR346c, and YPR145c-a are members of a small multigene family, sharing conserved KITRYDL and VITRHDL domains respectively. A triple mutation of these genes led to an observed decrease in membrane homeostasis, when cells were treated with the membrane disrupting detergents digitonin, ketoconazole, and

TTG. The same trends were observed when cells were exposed to the drugs cycloheximide, 4-NQ, SDS, and in the presence of the clinical antifungal drug amphotericin B. The observed phenotype suggests that this set of novel proteins regulates membranes in response to membrane-altering conditions, as an observed fifty-fold increase in membrane sensitivity of the triple mutant was observed for membrane acting compounds. For their observed phenotypes and apparent roles in the pleiotropic drug resistance network, the YGR035c, YLR346c, and YPR145c-a genes were respectively termed PDR19, PDR20, and PDR21. In order to help determine the molecular function(s) of these PDR proteins, a GAL4 two-hybrid system is being used to screen for proteins that may associate with the PDR 19/20/21 family proteins and help mediate their cellular functions.

THE YEAST TWO-HYBRID SYSTEM

In order to determine the functions and molecular activities of the PDR 19 and 20 proteins, the yeast two-hybrid system is being used to investigate their possible relationships with other proteins involved in membrane homeostasis. This technique allows for the identification of interacting proteins, by which a positive interaction signifies a similar cellular function and residence in a common protein complex. Aside from sequencing a protein of unknown molecular function, it is often equally important to identify interactions with other proteins as a next step in trying

to uncover its function(s). While it is largely believed that the suspect PDR proteins are involved in pleiotropic drug resistance, the yeast two-hybrid system will help identify interactions with other proteins.

In almost every process that occurs in the cell, there is an interaction between at least two, and often several, proteins. Protein interactions vary widely and occur in various processes such as transcription, translation, vesicular trafficking, membrane homeostasis, and cellular signaling. Protein-protein interactions play a pivotal role in normal cellular growth and homeostasis. In the case of protein modifications, for example, there is necessarily a protein-protein interaction based on both chemical and physical attractions. Protein kinases, glycosyl transferases, and phosphatases interact with their specific protein counterparts and allow for the transfer of a functional group from a specific amino acid^[6]. Several genetic diseases are often manifested through aberrant protein-protein interactions, such as sickle cell anemia. In the case of the pleiotropic drug resistance, the function of the PDR network is dependent upon gene products interacting with specific membrane proteins to elicit membrane homeostasis.

The yeast two-hybrid system involves the use of *in vivo* DNA transcriptional machinery. In particular, the system allows for the characterization of a protein of unknown molecular function based on an observed control protein interaction. The GAL4 gene and associated transcriptional machinery is what ultimately allows for the success of the

two-hybrid system. This constructed system makes use of two key concepts in gene transcription, regarding two DNA domains. The DNA binding domain recruits factors that bind directly to the promoter cis region, while the activation domain actually initiates transcription. In particular, transcription of GAL4 dependent promoters will not occur unless the DNA binding domain (DBD used in text to follow) is associated with a corresponding activation domain (AD used in text to follow). Interestingly, these two DNA domains do not need to be physically linked through covalent interactions^[6], thus allowing for the construction of a two-hybrid system. Fields and Song first proposed and demonstrated this mechanism through their use of SNF1 and SNF4^[6]. By creating fusion chimeras where SNF1 was fused to the DBD and SNF4 was fused to the AD, formation of a functional transcription factor was detected through phenotypic expression of the GAL4 reporter^[6]. This experiment proves that two related proteins will associate through non-covalent interactions. By fusing a “bait”/suspect protein to the DBD and a known prey protein/library to the AD, successful association leads to reporter gene expression through the transcription of the reporter GAL4 gene. In the event that the two proteins do not bind to one another, an interaction will not occur. Thus the AD will not associate with the DBD, which will not result in transcriptional activity.

As with any system, the two-hybrid system does have disadvantages. Firstly, the construction of a two-hybrid system involves

the transformation of a protein of interest fused to the DBD into a yeast strain containing a reporter gene. If the fusion protein causes transcription on its own, it is said to autoactivate the reporter. In this particular case, the protein cannot be used as an effective “bait” in screening a library of AD fusion cDNA clones^[6]. With any system that involves the use of chimeras, it is always possible for the native quaternary protein conformations to become compromised during genetic manipulation. If the native conformations of the bait or prey protein are changed, it is quite likely that the proteins may not interact as they normally would do so *in vivo*. Another apparent disadvantage in using the two-hybrid system is the obvious fact that protein-protein interaction occurs in yeast cells. In studying proteins from other organisms by this system, it is essential that they be able to fold correctly in yeast^[6], as failure to do so will undoubtedly disturb native covalent interactions. Yet another disadvantage with the two-hybrid system is that the only measurable indication of interaction is the phenotypic expression of the reporter gene. Although fairly unlikely, it is possible that a third protein could bridge the two fusion proteins, thereby initiating transcription of the reporter^[6].

Most of the aforementioned disadvantages are minor when considering the biological value of the system’s advantages. Perhaps the most obvious advantage is the fact that the two-hybrid system employs the use of DNA transcriptional domains and associated machinery. Unlike traditional biochemical approaches, protein-protein interaction in this

construct occurs *in vivo*. Since detection does not occur *in vitro* or through bacterial expression, low affinity protein-protein interactions are noted more frequently by transcription of the reporter^[6]. In other words, there is a significant degree of amplification in the form of the reporter, whereas some biochemical assays may not necessarily isolate these transient interactions. Perhaps the greatest advantage of the system is that a known protein interactor can be used to functionally characterize an unknown protein in an *in vivo* environment. This property makes the yeast two-hybrid system an effective tool in characterizing cascade signaling or even targets of cellular networks, such as the pleiotropic drug resistance network. The speed and relative ease in creating fusion proteins makes this system an ideal tool in the characterization of protein interaction.

**EXPERIMENTAL APPROACH: Use of the GAL4
two-hybrid system to attempt to identify proteins
associating with PDR 19 and PDR20**

Perhaps one the most critical decisions in designing a two-hybrid system is the choice of the vector system. The most frequently used DBD and AD containing vectors are based in the GAL4 system, as the reporter gene activity is easily evaluated. However, in some cases other vectors are used, such as the *LexA* system^[6]. Nonetheless, it is important to use a bait vector with certain features. In this case, the pAS II vector was used. The pAS II plasmid contains a hemagglutinin epitope tag, inserted in the GAL4 DBD reading frame^[6], which allows for HA protein tagging and

visualization by immuno-blot. Most plasmid vectors in yeast two-hybrid systems also include a selectable nutritional marker, as is the case with the TRP1 gene in pAS II. This ultimately allows for selection upon transformation on synthetic complete media in the absence of tryptophan. The pAS II plasmid vector used in construction of the PDR two-hybrid system is of the following type: DBD_{GAL4} HA epitope, TRP1. In the case of the “prey”/library plasmid vector, pACT II was used, which contains the GAL4 activation domain. As with pAS II, pACT II also allows for nutritional selection, but is selective on synthetic complete media minus leucine. Specifically, pACT II consists of the following selectable markers: AD_{GAL4} HA epitope, LEU2.

One obstacle that the GAL4 yeast two-hybrid system must circumvent is the inherent behavior of GAL4 and GAL80 in the presence or absence of galactose. As is the case with all yeast genes, a functional TATA box is located upstream of the gene. The promoter region of a functional gene typically includes the TATA box and its associated cis-acting transcriptional elements. One of the most prevalent cis-acting transcriptional elements present are the upstream activating sequences (UAS). These sequences in yeast bind two regulatory proteins: Gal4p and Gal80p. These two regulatory proteins control galactose metabolism. In the presence of galactose, Gal4p binds to the GAL elements within its corresponding UAS^[6]. However, in the absence of galactose, Gal80p binds to Gal4p, thereby blocking transcriptional activity. Obviously, in

constructing a yeast two-hybrid system, it is necessary to avoid this natural interaction between the two GAL proteins. To do so, the two-hybrid system must contain deletions of the GAL4 and GAL80 genes, which consequently results in slower growth^[6].

In order to construct the PDR 19/20 two-hybrid system, the following yeast strains were used: YSE859 Y190; MAT α gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 URA3::Gal-lacZ, Lys2::GAL(UAS)-HIS3 and YSE32 Y187; MAT α gal4 gal80 his3 trp1-901 ura3-52 leu2-3,-112 URA3::GAL-LacZ.

The yeast strain Y190 was transformed with the plasmid pAS II and PDR19 and 20 clones: YLR B-1, YLR A-4, YGR A-2, and YGR B-1. This was done using a standard plasmid transformation into yeast protocol. 2mL of Y190 yeast cells in YPD liquid media were grown overnight at 30°C in an incubator. 500 μ L of cells were spun down and the liquid media was aspirated. 100 μ L of one-step buffer (1mL stock: 200 μ L 2M LiAc, 800 μ L 50% PEG, and 7.69 μ L β -Mercaptoethanol) was added. 5 μ L of salmon sperm DNA were added with 1 μ L of the “bait” plasmid, pASII. As mentioned, pAS II contains the DBD_{GAL4}. After heat shocking for 30 minutes, the cells were plated on synthetic complete media minus tryptophan. The pAS II carries TRP1 as a selectable nutritional marker. Transformants carrying the plasmid will therefore be able to grow on the SC-Trp media.

The Y187 strain was successfully transformed with pACT II, containing the AD_{GAL4}, using the transformation protocol. Since pACT II contains a LEU2 nutritional selection marker, the cells were plated on SC-leu media. Rather than co-transforming the two fusion strains onto a SC-leu-trp plate, a modified replica plating technique was used. The Y190 transformants were patched onto a SC-leu-trp plate and physically mated with the Y187 strain, containing the pACT II activation domain plasmid. Upon incubation at 30°C, for two days, positive growth was noted on the SC-leu-trp plate signifying a successfully constructed two-hybrid system (See Appendix1). Eventually, a library screen will be carried out, searching for proteins that interact with the novel PDR 19 and PDR 20 proteins. Isolating such protein interactions will increase understanding as to what the exact molecular functions of these novel proteins are and how they participate in membrane homeostasis.

CONFIRMATION of NEGATIVE AUTOACTIVATION TESTS

The constructed yeast two-hybrid system is largely based on the transcriptional activity of the reporter GAL4 gene. Since the system involves the control clones and the “bait” pAS II DNA binding domain plasmid, it is important to make sure that transcription is in fact due to the interaction between two interacting proteins and not DBD fusion activation. The Y190 strain used in the early transformations contains a reporter gene used to assess autoactivation, namely the HIS3 gene. Activation of the

reporter would be due to autoactivation by the DBD/ PDR19 and/or 20 fusions, thereby initiating transcription of the reporter gene.

The presence of the reporter HIS3 gene allows for nutritional selection on SD media in the presence of varying concentrations of the HIS3 protein competitive inhibitor, 3-aminotriazole. Following collection and cultivation of independent transformants for the bait plasmids, autoactivation was studied by observing growth on SD-His media. Theoretically, an increase in the concentration of the histidine enzyme should lead to an increase in the concentration of histidine even in the presence of the 3-aminotriazole competitor, hence growing on SD+40mM aminotriazole. This of course reflects activation by the DBD-fusions, and is thereby indicative of autoactivation in the constructed GAL4DBD-PDR fusion proteins.

In order to assess for autoactivation in the PDR19/20 yeast two-hybrid construct, growth relative to known interactors were compared. For the positive growth control, the YSE1340 strain (Y190 carrying an Erdman lab constructed bait plasmid PXL1-DBD) plus OSE3-AD was patched on SD + 40mM aminotriazole and incubated for 24 hours. This yielded positive growth on the media, as activation of the GAL-HIS3 promoter lead to an increase in the concentration of the His enzyme, thereby increasing the concentration of free histidine. For the negative growth control, YSE1340 plus the pACT II carrying the empty AD was assessed. After 24 hours of incubation, no growth was observed. In the mated Y187/859

(containing PDR/pACT II) no activation of the promoter was observed, and hence no observed growth. Upon patching on SD+40mM aminotriazole, there was no observed growth for all Y187/859 strains containing the PDR19/20 baits and empty activation domain vector (See Appendix 1b). This indicates that these fusions can be used for future screens using the system.

Currently, plasmid sequencing of the GAL4DBD-PDR fusion proteins is in process to help confirm proper cloning of the bait proteins prior to library screening (See Appendix 2).

FUNSPEC ANALYSIS of TTG RESISTANCE and SENSITIVITY AMONG NON-ESSENTIAL *S.cerevisiae* GENES

In studying the composition of cell membranes, at least two unanswered questions can target the direction of research. First, how do fungi cope with naturally occurring products present in their environments, which may be toxic to them through affecting their cell membranes? Many such natural products are thought to exist in plants as defense mechanisms. Secondly, how do eukaryotic cells in general maintain membrane homeostasis in the event of environmental challenges? The yeast genome is comprised of roughly 6,000 genes. Nearly 30% of yeast ORF's encode membrane proteins, a statistic that should come at no surprise considering the number of different compartment membranes in eukaryotic cells. Every compartment membrane has a genetically

controlled mechanism that maintains membrane homeostasis. Currently, there are approximately 900 yeast ORF's with unknown functions, some of which could function in membrane homeostasis. This appears to be the case for the recently isolated family of proteins PDR 19/20/21.

One of our aims is to understand how eukaryotic cells maintain membrane homeostasis in the face of environmental challenges. Of the many natural products fungi encounter, some glycosides of triterpenes are known to present a possible challenge to membrane integrity and overall homeostasis. Triterpenes are natural C₃₀ compounds, whose glycosides are saponins that occur in plants as a possible defense compound. TTGs disturb membranes through such mechanisms as sterol binding, often rendering the membrane more permeable. Studying membrane disturbing compounds and their associated effects on membrane disruption allows for further insight into both the pleiotropic drug resistance network and how vesicular trafficking proteins maintain membrane homeostasis. Interestingly, wild type yeast backgrounds differ in resistance to TTGs. For instance, the wild type W303 strain is sensitive, while BY4743 and its derivatives are not as sensitive. In a previous screen in the Erdman lab, 4,851 deletion strains of the BY4743 background were screened, of which 991 strains demonstrated a degree of sensitivity or resistance to TTGs relative to the wild type strains. Of the 991 strains, 110 were supersensitive, 276 were sensitive, 407 were moderately sensitive, and 53 deletion strains were weakly sensitive. A total of 131 resistant strains

were isolated. Interestingly, it is estimated that 80% of the TTG phenotypes are linked to kanMX. KanMX was systematically used to replace ORFs, in essence establishing an ORF knock-out collection. The results from the deletion screen are of interest, for there must be an underlying mode of genetic control governing the range of observed phenotypic sensitivities. In an attempt to further understand and classify these results, a bioinformatics tool, Funspec (T. Hughes Lab, U. Toronto) was used^[2]. Funspec is a program that queries yeast databases with an input set of genes (in our case the genes whose deletion leads to TTG sensitivity or resistance), and compares their MIPS (Munich Information Center for Protein Sequences) and GO (Gene Ontology) classifications to those of all the genes present in the yeast genome. Such classifications take into consideration the molecular function, cellular component, subcellular localization, and protein complexes.

TTG RESISTANCE

In querying yeast databases with an input set of genes whose deletions confer resistance, there were several common MIPS and GO classifications that perhaps offer insight into drug resistance and cellular processes controlling membrane homeostasis. Interestingly, the greatest possibility value (p) for GO biological processes showed that most genes are involved with the sterol biosynthetic process, phospholipid translocation, and steroid biosynthetic processes in general. This finding

is consistent with the notion that TTG resistance employs alternative proteins of sterol biosynthetic pathways, and perhaps thereby physically resists alterations in the lipid bilayer. Not surprisingly, one of the main targets of TTGs is in fact the bilayer integral sterol molecule. Sterols, among other functions, are responsible for controlling the fluidity of the lipid bilayer. TTG targeting of sterol molecules disturbs membranes through a variety of mechanisms, most notably TTG complexes to ergosterol. The following ergosterol genes are involved with resistance: ERG2, ERG3, and ERG6, perhaps conveying resistance to TTGs (See Appendix 3a).

Additionally, several of the TTG resistant genes are involved with phospholipid translocation. DRS2, DNF2, and LEM3 all function to maintain membrane homeostasis. DNF2 and DRS2 are both aminophospholipid translocases or flippases that are involved in endocytosis, protein transport, and cell polarity. Both are type 4 P-type ATPases. KES1 and CYB5 are also involved with membrane homeostasis and TTG resistance. KES1 is a member of the oxysterol binding protein family that regulates Golgi complex secretory functionality. Like the other proteins in its functional class, Kes1p functions on the cytosolic side of the plasma membrane. In the case of CYB5, Cytochrome b5, it is involved in sterol biosynthetic pathways, and specifically donates electrons to support C5-6 desaturation^[2].

As previously mentioned during the discussion of the PDR network, plasma membrane resistance is often induced by signals produced from dysfunctional mitochondria. Loss of the inner mitochondrial protein Oxa1p results in a generated signal that increases the concentration of PDR5, a membrane efflux pump of the ABC superfamily^[3]. In other cases, substitution mutations within the PDR1 or PDR3 proteins result in observed pleiotropic drug resistance, by again increasing the concentration of the PDR5 efflux pump protein. Not surprisingly, when looking at the bioinformatics of cellular respiration and mitochondrial function involved in TTG resistance, almost all genes encode proteins that are functionally involved in processes that occur within the inner membrane of the mitochondria. IMP1, CYC3, and COR1 are three prime examples of this observation. IMP1 is a gene whose protein functions in the catalytic subunit of the mitochondrial inner membrane peptidase complex^[2]. In the case of COR1, the protein functionally resides in the subunit of the ubiquinol-cytochrome c reductase complex, and is again a component within the inner membrane electron transport chain^[2].

Aside from cellular functions involving membrane phospholipids, sterol biosynthesis, and mitochondrial functionality, TTG resistance also seems to be characterized by changes in cell polarity and cellular signaling. Genes involved in these processes include membrane protein genes such as LEM3 and cdc42p-activated signal transducing kinases such as STE20. When considering all of the genes above, it becomes

clear that TTG resistance is not controlled by any one particular gene, but rather is a collaborative genetic product of several networks. Such networks include ergosterol biosynthesis, mitochondrial functionality, PDR, YAP, and cellular signaling. Additionally, several novel genes of unknown function have been found as a result of both TTG screens and bioinformatic approaches. Such genes include YPL191c, YBL089w, YFL006w, YBR255w, YKL023w, and YNR047w. It is possible that one or more of these genes function in one of the key genetic pathways mentioned.

TTG SENSITIVITY

Upon evaluating the TTG sensitive genes, an interesting trend develops. Although many of the cellular functions fall under the same category, the individual genes involved differ from those expressed in TTG resistant phenotypes. Consider TTG sensitive genes involved in membrane phospholipids and sterols. Unlike genes involved in resistance, a different set of ergosterol genes are involved in sensitivity. Particularly, the following ergosterol genes are involved: ERG4 and ERG5. Additionally, OSH3 is involved with membrane sensitivity (See Appendix 3b). Interestingly, TTG sensitivity employs the use of OSH3, an oxysterol-binding protein involved in sterol metabolism, whereas TTG resistance entails the expression of OSH4/KES1. OSH4/KES1 is involved in the negative regulation of the Sec14p, which is a phosphatidylcholine transfer protein. OSH4 de-regulates Golgi vesicular trafficking that is

regulated by the Sec14p^[2], and overall perhaps influences lipid distribution within the membrane.

Interestingly, TTG sensitivity also returned genes involved in cell wall assembly and stress response proteins. Such is the case with SFL1, which is an activator of stress response genes^[2]. Likewise, GAS1 is a gene required for cell wall assembly, as its protein specifically functions as a glycosphospholipid-anchored surface protein. Perhaps one of the most interesting observations concerns the killer toxin resistance genes: FYV4, FYV6, and FYV12. These genes, when deleted, cause TTG sensitivity, even though the precise molecular functions of these proteins are currently unknown. It appears, though, that these genes are activated in response to K1 killer toxin. Perhaps the activation of these genes is in response to a disturbance that mimics TTG membrane disruption. In fact, KI is a perforating protein that destroys sensitive yeast cells^[2]. In this case, it appears that the FYV genes are not effective in resisting membrane disruptions created by TTGs, but are activated as a result of the yeast cell's overall sensitivity. In resistant strains it was noted that PDR genes and sterol biosynthetic pathways were activated.

Additionally, in TTG sensitivity, mitochondrial function and respiration cellular functions are not observed. This offers further support that sensitive strains do not activate the PDR1/3 genes through mitochondrial signaling, which results in overexpression of the ABC type PDR5 efflux pump. If the functional classes of genes enriched in the TTG

screen are compared between the resistant loci and sensitive loci, several differences are noted. Generally, TTG resistant loci encode ribosomes, lipids, isoprenoids, and sterols. TTG sensitivity-causing loci involves functional classes such as transport ATPases, lipids, sterols, and ion/pH homeostasis. When considering these two groups of functional classes, a trend becomes apparent. TTG resistance generally involves ribosomal machinery and translational events, suggesting that there is an active attempt by the cell to express gene products that operate to maintain membrane homeostasis. Moreover, different ergosterol pathways are used to resist TTG membrane disturbances. With TTG sensitivity, different functional classes are involved, in particular ATPases. As previously mentioned, TTGs complex with sterols and perhaps disrupt ATPase activity. Curiously, sensitivity maybe related to cellular attempts to replenish ATPase activity, while a degree of resistance entails a different sterol biosynthetic pathway to produce un-complexed sterols. While this has not been proven, there is strong supporting bioinformatic evidence, as will be discussed in the next section.

ERGOSTEROL BIOSYNTHESIS in RESPONSE to TTG

In analyzing the results of the two queries involving TTG resistance, some mechanisms through which membrane homeostasis may be disrupted become evident. As illustrated in the results above, TTG can associate with ergosterol, possibly leading to an increase in ergosterol

concentration by the cell as a compensatory mechanism. Interestingly, different components of the ergosterol biosynthetic pathway are involved in the resistant strains, which suggest possible resistance due to byproduct formation. Inactivation of ERG3/6/2 and KES1 cause phenotypic TTG resistance, while ERG4/5 and the oxysterol binding protein OSH3 lead to sensitivity.

A closer investigation of the ergosterol biosynthetic pathway reveals that genes involved in TTG resistance may produce enol byproducts, which could lead to an increased production of sterols. Specifically, ERG 6 is capable of producing dienol, trienol, and tetranol byproducts. ERG2 likewise produces enol, dienol, and trienol forms. Lastly, ERG3 produces byproduct sterols existing in both enol and dienol forms.

When considering these results, it becomes apparent that while TTG complexes with ergosterol, sensitive strains may only activate ERG5/4, which consequently increases the concentration of ergosta-5,7,22-trienol (ergosterol). The ergosterol molecules produced are identical to the integral ergosterol molecules within the bilayer, resulting in further complexing by TTGs. In resistant strains, the ergosterols are not of the ergosta-5,7,22-trienol form, and exist in the enol byproduct forms previously mentioned. Interestingly, in resistant strains the concentrations of these byproduct enol forms increase upon exposure to TTG, suggesting that these forms are perhaps incapable in complexing with TTG, thereby

leading to increased phenotypic resistance. Sensitive strains cannot produce any other form of ergosterol, which perhaps leads to the observed sensitivity.

In a related observation, azole drug mediated fungal targeting currently seeks to target ERG11. Azole targeting in yeast targets a cytochrome P450 which is a C14 α -demethylase, encoded by ERG11. Targeting the gene product of ERG11, namely C14 α -demethylase, confers sensitivity. Resistance, however, can occur via an alternative pathway of a mutated ERG3 gene. In this case, normal sterol production is altered during subjection to the antifungal drug, consequently developing a deficiency in a sterol desaturase, and the cell thereby resists membrane degradation. Another focus involves further analysis of the ERG4 gene, one of the final genes in the pathway, responsible for the sterol C-24 reductase. Upon deletion of this specific gene, ergostan 5,7, 22, 24-tetraen-3 β -ol, a precursor to ergosterol, accumulates within the membrane, which might explain the observed drug hypersensitivity.

Interestingly, our investigation pertaining to the suppression screens used with TTG to analyze compensatory mechanisms in the absence of ergosterol produces similar drug resistance patterns as with previous antifungal tests. This, once again, suggests that there is a broad cellular response to drugs that act as membrane disturbing compounds.

CONCLUDING REMARKS

The plasma membrane is one of the most critical structures in cells. Through regulation and homeostatic measures, the membrane resists environmental challenges, such as naturally occurring toxic compounds. In an attempt to further understand the underlying genetic mechanisms, recent genetic screens in yeast for plasma membrane homeostatic proteins identified three related proteins of unknown molecular function that participate in these processes. These proteins, termed PDR19, PDR20, and PDR21 for Pleiotropic Drug Resistance, are each approximately one hundred amino acids in size and share a small conserved domain. The coding genes for this set of proteins may be found in the ORFs YGR035c, YLR346c and YPR145w-a, respectively. Functionally, these proteins appear to be effectors of the PDR network, as a triple mutation of these genes led to an observed decrease in membrane homeostasis, when treated with the membrane disrupting reagents. The observed phenotype suggests this set of novel proteins acts to functionally resist/regulate membranes in response to membrane-altering conditions. Further analyses suggest a functional role not only in chemical resistance, but perhaps also in membrane composition.

(i) In order to determine the functions and molecular activities of the PDR proteins, the yeast two-hybrid system will be used to investigate their relationship with other related proteins involved in membrane homeostasis. This technique allows for the identification of interacting

proteins/components, and is also a tool to manipulate native interactions. A yeast two-hybrid system containing the PDR19/20 proteins has successfully been constructed, although screening for interactors has not yet occurred. That this system can be used has been confirmed through negative autoactivation tests involving a related protein from a modified Y187/Y190 mating strain and the pACT II activation domain plasmid containing the ADGal4. Plasmid sequencing of the Gal4DBD-PDR fusion proteins is in process to help confirm proper cloning of the bait proteins in addition to library screening.

(ii) One of the many aims in studying membrane homeostasis is to further understand resistance and sensitivity associated with exposure to naturally occurring toxic compounds. Of the many natural products, fungal cells may encounter some glycosides of triterpenes are known to present challenges to membrane integrity and overall homeostasis. In a screen for the effects of TTG membrane disruption, 4,851 yeast deletion strains were screened, of which 991 demonstrated a degree of sensitivity or resistance. In attempt to categorize and further understand the genetic components involved with these phenotypes, a bioinformatic tool was employed. Funspec analysis demonstrated that there are distinct genetic candidates and pathways involved in mediating resistance or sensitivity to membrane disturbing compounds. Most notably, the ergosterol biosynthetic pathway and PDR networks were synonymous with phenotypic resistance.

Understanding the homeostatic processes and resistance patterns involved with drug challenges holds significant clinical importance. Fungal infections are especially devastating to pediatric patients and those with immunosuppressed systems, such as AIDS patients. Through an understanding of the biosynthetic pathways essential for membrane homeostasis, it is quite possible that novel non-invasive treatments for fungal infections may be developed. Such antifungal activities would target specific components and pathways essential for membrane stability. Identifying and isolating the novel proteins involved in these pathways is an important step in developing such treatments. In addition, such studies also lead to a greater understanding of our own cells. When we consider that one of the greatest challenges in drug design today is cellular uptake of compounds by the plasma membrane, even the smallest of discoveries may one day have a much more profound impact. Our findings regarding plasma membrane homeostasis mechanisms will contribute to a much larger collection of biomedical discoveries that are medically important to drug design and delivery mechanisms.

SOURCES CITED and CONSULTED

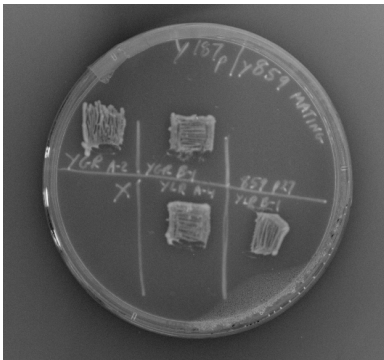
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APPENDICES

Appendix 1:

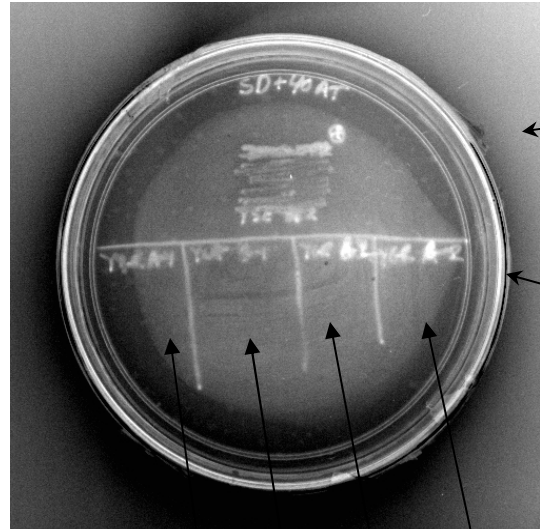
a.)



Generation of a modified Y187/Y190 mating strain and the pACT II activation domain plasmid containing the ADGal4. Yeast strain Y190 was transformed with the “bait” plasmid pAS II and control clones: YLR B-1, YLR A-4, YGR A-2, and YGR B-1 on synthetic complete (SC) media minus Trp. Using a modified replica plating technique, the Y190 transformants were successfully mated with Y187 strain, containing the pACT II activation domain plasmid, allowing growth on an SC-Trp-Leu plate.

b.)

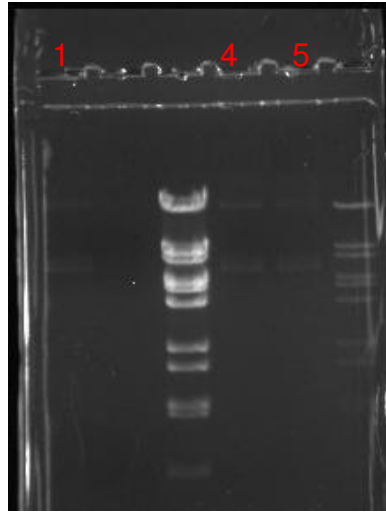
Evaluation of Autoactivation



Positive growth on SD + 40 mM aminotriazole [Y1340+p507 (Pxl1p-Dse3 interaction)]

No growth of all Y187/859 strains containing the PDR19 and PDR20 baits and an empty activation domain vector

YGR A-2
YGR B-1
YLR B-1
YLR A-4

Appendix 2:

YLR B-1 pASII (1)
YGR A-2 pASII (4,5)

Gel electrophoresis showing plasmid DNA containing the GAL4DBD-PDR fusion proteins. Future plasmid sequencing will help to confirm proper cloning of the bait proteins.

Appendix 3:

a.)

Key Funspec Analysis of TTG Resistant Genes

P<0.005 **k**: number of genes from the input cluster in given category.**f**: number of genes total in given category.

MIPS Functional Classification

Category	p-value	In Category from Cluster	k	f
Ribosomal Proteins	5.91e-05	RPL19B RPS8A RPL23A RML2 RPL24B RPS24B RPS21B RPL14A RPL8B RPL6B MRPL39 RPS18B RPS10B RPS7B RPP2A	15	246
Tetracyclic/pentacyclic triterpenes metabolism	0.004513	ERG3 ERG6 ERG2 KES1	4	36

GO Biological Process

Sterol biosynthetic process	0.0002188	ERG3 ERG6 ERG2 CYB5 KES 1	5	30
Phospholipid translocation	0.001286	DRS2 DNF2 LEM3	3	20
Steroid biosynthetic process	0.001325	ERG3 ERG6 ERG2 KES1 STE14 STE20	4	26

GO Cellular Component

Ribonucleoprotein complex	0.0001149	RPL19B RPS8A RPL23A RML2 NOP16 RPL24B	17	324
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b.)

Key Funspec Analysis of TTG Sensitive Genes

MIPS Functional Classification

<u>Category</u>	<u>p-value</u>	<u>In Category from Cluster</u>	<u>k</u>	<u>f</u>
Homeostasis of protons	0.0006376	PPA1 VMA5 VPH2 VMA6 ATP15	5	47
Tetracyclic/pentacyclic triterpenes metabolism	0.001938	ERG4 OSH3 YEH2 ERG5	4	36

GO Biological Process

Double-strand break repair via nonhomologous end joining	0.001239	RSC1 RAD27 FYV6 RAD50	4	32
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GO Cellular Component

proton-transporting two-sector ATPase complex	0.0004013	PPA1 VMA5 VMA6 ATP15	4	24
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