Identifying the Amino Acid Residues Responsible for Molecular Diode Function in Pdr5, a Major Multidrug Efflux Pump

A DISSERTATION
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By
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Identifying the Amino Acid Residues Responsible for Molecular Diode Function in Pdr5, a Major Multidrug Efflux Pump

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Director: Dr. John E. Golin, Ph.D.

Antimicrobial resistance (AMR) occurs when a microorganism gains resistance to antimicrobial agents that were originally designed to cure infections caused by these microorganisms. Overexpression of ATP-binding cassette (ABC) multidrug transporters is a mechanism of AMR, which acts by pumping the drugs out of the cell. ABC transporters are a superfamily of polytopic proteins that uses the energy of ATP hydrolysis to transport the substrates across the membrane against a concentration gradient. The typical ABC transporters consist of two transmembrane domains (TMDs), composed of six transmembrane helices (TMHs) that are connected by extracellular loops (ECLs) and intracellular loops (ICLs), and two nucleotide-binding domains (NBDs).

ABC transporters export substrates through a series of conformational changes. Many ABC transporters shift between an inward-facing, drug-binding (high-affinity) conformation to an outward-facing, drug-releasing (loose-binding) conformation using ATP binding and hydrolysis. When the drug is released from the drug-binding pocket, it must be prevented from reentering through the transporter. The outward-facing or drug-releasing conformation results in only a 10-to-30-fold reduction in binding affinity, which is not sufficient to prevent some rebinding of the drugs if the extracellular concentration is high (Sauna and Ambudkar, 2000). For this reason, it was proposed that ABC transporters have a molecular gate or diode that prevents reflux (Gupta, et al., 2011).
The first evidence for a molecular diode mechanism was the observation that a Ser 1368 Ala (S1368A) mutation in the yeast multidrug transporter Pdr5 exhibited significant drug reflux during transport (Mehla, et al., 2014). More recently, multiple cryo-electron microscopy structures of the ABCG2 mammalian multidrug transporter clearly showed the presence of a molecular gate between an inner and outer drug-binding pocket. Two prominent residues—Leu-554 and Leu-555—were identified as constituents of this gate (Manolaridis, et al., 2018). When the sequences of Pdr5 and ABCG2 are aligned, Ser-1368 is quite close to Leu-554 and Leu-555. The corresponding residues in Pdr5 are Val-1372 and Met-1373. Bioinformatics analysis of the Pdr subfamily (Lamping, et al., 2010) revealed highly conserved residues adjacent to Ser-1368, including Phe-1369, Gly-1371, Val-1372, and Met-1373. The corresponding residues to this S1368 adjacent motif in the amino-terminus of Pdr5, extending from Tyr-680 to Ilv-685, also exhibit conservation.

The first goal of this study is to determine whether the conserved set of amino acids spanning residues (1369–1373) in the carboxyl-terminus motif of Pdr5 make up a molecular diode motif. The second goal is to investigate whether the molecular diode is composed of residues from both halves of this large, polytopic protein. Our results suggest that this conserved motif plays a variety of roles including signal transmission, protein folding, and molecular gating. Our results also suggest that Ile-685, Val-1372, and Met-1373 are the actual gate, where Ser-687, Ser-1368, and Phe-1369 provide structural support. Alanine substitutions in these residues exhibit a unique gating-defect phenotype. Characterization of mutants in these two motifs might lead to specific chemical inhibitors of these transporters during disease treatment.
This dissertation by Maryam Alhumaidi fulfills the dissertation requirement for the doctoral degree in Cell and Microbial Biology approved by Dr. John E. Golin Ph.D., as director and by Dr. John Choy Ph.D. and Dr. Ekaterina Nestorovich Ph. D. as readers.

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<tr>
<td>5-FOA</td>
<td>5-Fluoroorotic acid</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ADP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ALDP</td>
<td>Adrenoleukodystrophy protein</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin (Amp)</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<tr>
<td>clo</td>
<td>Clotrimazole</td>
</tr>
<tr>
<td>Cyc</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECL</td>
<td>Extracellular loops</td>
</tr>
<tr>
<td>G-418</td>
<td>Geneticin- 418</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IAAP</td>
<td>Iodoarylazidoprazosn</td>
</tr>
<tr>
<td>ICL</td>
<td>Intracellular loops</td>
</tr>
<tr>
<td>IFIs</td>
<td>Invasive fungal infections</td>
</tr>
<tr>
<td>LB</td>
<td>Lauria-Bertani</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>MQ</td>
<td>MilliQ</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug-resistance protein</td>
</tr>
<tr>
<td>NBDs</td>
<td>Nucleotide-binding domain</td>
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PCR                  Polymerase Chain Reaction
PDR                  Pleiotropic drug resistance
Pgp                    P-glycoprotein
Pi                       inorganic phosphate
PM                     plasma membrane
R6G                    rhodamine 6G (R6G)
RLI                    RNase L inhibitor
RPI                    Research Products International (RPI)
rpm                   revolutions per minute
SD+his              Synthetic dextrose with histidine
SDS-PAGE         Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE                  Tris acetate acid EDTA
TAP            Transporter associated with antigen processing
TMDs              Trans-membrane domains
TMH                   Transmembrane helix
TMS                   Transmembrane spans
Vmax                Maximum rate achieved by enzymatic system
WHO                 World Health Organization
WT                     Wild type
YEF3                 Yeast elongation factor EF-3
YPD                  Yeast peptone dextrose
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My kids Abdulllah, Laura, and Dina, you are behind my achievement.
INTRODUCTION

Antimicrobial resistance:

Antimicrobial agents are widely used in the treatment of infectious diseases caused by bacteria, yeast and parasites. These drugs work by inhibiting or interfering with a variety of cellular processes including membrane and cell wall biosynthesis, DNA replication, and protein synthesis. Since the 1940s, antibiotics successfully reduced the deaths caused by infectious diseases. However, the emergence of antimicrobial resistance has made treatment less effective and has become a major clinical challenge. According to the Center for Disease Control and Prevention (CDC) report, more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result (CDC, 2019).

The evolution of AMR strains can occur through a process known as horizontal gene transfer—when genetic material conferring AMR is transferred directly between bacterial strains. AMR can also occur spontaneously through random mutation (Alexopolous, 1996). Data from World Health Organization (WHO) report has shown high rates of cephalosporin resistance and fluoroquinolone resistance in *Escherichia coli* (*E. coli*), cephalosporin resistance and carbapenem resistance in *Klebsiella pneumoniae*, and methicillin resistance in *Staphylococcus aureus* (WHO, 2018). Patients infected with resistant bacterial strains require more health-care resources than patients infected with non-resistant strains of the same bacteria (WHO, 2018).

AMR is not limited to bacteria. AMR is also observed in a significant percentage of parasites, including: *Plasmodia, Leishmania, Entamoeba, Trichomonas vaginalis,*
schistosomes, and Toxoplasma gondii, against drugs such as chloroquine, pyrimethamine, artemisinin, pentavalent antimonials, miltefosine, paromomycin, and amphotericin B as well as atovaquone and sulfadiazine (Tanwar, et al., 2014). The development of resistance to antimalarial drugs is arguably the greatest threat to malaria control and results in increased malaria morbidity and mortality.

Even though bacteria, followed by parasites, are responsible for most severe human infections, fungi can also cause fatal infection. Fungi can cause major morbidity and mortality in immunocompromised patients by triggering superficial infections of the skin or mucosal surfaces and they can disseminate hematogenously (Lamping, et al., 2010). The severity of fungal infections ranges from mild to fatal depending on the site of infection and the immune condition of the patient. Fungal penetration into epithelial surfaces of immunocompromised patients can cause invasive fungal infections (IFIs). The most common causative agents of IFI are Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans (Lamping, et al., 2010).

The development of antifungal drugs is especially challenging because fungi are eukaryotic cells and have many similarities with the cells of human hosts that they infect. However, it has been possible to exploit some metabolic differences to develop antifungal drugs. Polyenes such as Amphotericin B and Nystatin B were developed as the first generation of antifungals. Polyenes target ergosterol; a cell membrane component that is analogous to cholesterol in mammalian cell membranes. Nevertheless, these compounds were still highly toxic to humans. Two decades later, azoles were developed and made a major breakthrough as antifungal therapy. The azoles target the biosynthetic pathway of ergosterol by inhibiting an early-phase enzyme called lanosterol 14α-
demethylase encoded by the \textit{ERG11} gene. This leads to the formation of toxic sterol that inhibits further fungal growth. However, the fungistatic nature of the drug combined with their ability to interact with cytochrome P450 enzymes created differences in the treatment of patients undergoing multidrug therapy. In 2001, echinocandins were discovered as promising drugs to treat IFIs. Echinocandins are fungicidal drugs that block cell-wall synthesis by inhibiting \(\beta-(1, 3)\)-D-glucan synthase (Srinivasan, et al., 2014).

Even though there is only a limited number of antifungal drugs available for the treatment of fungal infections, resistance to antifungal drugs such as polyene macrolides (amphotericin B), azole derivatives (ketoconazole, fluconazole, itraconazole, and voriconazole), DNA and RNA synthesis inhibitors (flucytosine), and 1,3-\(\beta\)-glucan synthase inhibitors (echinocandins) exists in \textit{Candida spp.}, \textit{Aspergillus spp.}, \textit{Cryptococcus neoformans}, \textit{Trichosporon beigelii}, \textit{Scopulariopsis spp.}, and \textit{Pseudallescheria boydii}. Antiviral drug resistance was also observed in immunosuppressed transplant recipients and oncology patients infected by either cytomegalovirus (CMV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), influenza A virus, hepatitis C (HCV), or hepatitis B virus (HBV) (Tanwar, et al., 2014).

\textbf{Mechanisms of antimicrobial resistance:}

Antibiotic resistance is estimated to cause around 300 million premature deaths by 2050, with a loss of up to $100 trillion (£64 trillion) to the global economy (Munita and Arias, 2016). Understanding the mechanisms behind multidrug resistance is important if it is to be eliminated. Resistance to antimicrobial agents arises through
several methods, including alteration of drug uptake or drug target, sequestration of the
drug, drug inactivation, or the active efflux by overexpressed transporters (Figure 1).

**Figure 1: Mechanisms of AMR.** There are various ways by which pathogenic organisms
develop resistance to therapeutics including: impaired uptake of the drug, modification or
increased expression of cellular drug targets, drug inactivation, and active efflux by
overexpressed transporters.

Genetic changes in the enzymes mediating ergosterol biosynthesis, a major
component of membranes, are a major cause of drug resistance in pathogenic fungi.
These enzymes are targeted by azoles, which are a major class of drugs used to treat
infection. Change in ergosterol biosynthesis has been shown in some species of *Candida*
as a mechanism of resistance to widely used azole antifungals, which requires direct interaction with structural cellular components of the fungal cell membrane. The target of azole antifungals is the cytochrome P450 enzyme 14α-lanosterol demethylase, which is essential to ergosterol biosynthesis. This enzyme is encoded by ERG11 (also referred to as CYP51). Inhibition of Erg11p reduces the synthesis of membrane ergosterol and results in the accumulation of toxic sterols that inhibit fungal growth. Point mutations in Erg11p can cause a reduction in azole binding (Lamping, et al., 2010). The probable causes of resistance are the alteration in 14α-lanosterol demethylase by either mutation or overexpression. This mechanism has also been observed in *C. glabrata* and *C. albicans* (Ghannoum and Rice, 1999). Alterations in fungal membrane composition including β-1,3-glucan, and lipid content reduce binding sites for echinocandins, i.e. reduce the amount of drug getting into the cells (Ghannoum and Rice, 1999).

The major mechanism responsible for high levels of azole resistance in fungi such as *Candida sp.*, however, is the overexpression of efflux pumps. These transmembrane proteins pump the drug out of the cell, reducing intracellular azole concentrations to levels at which ERG11p is not inhibited. Azole-resistant *C. albicans* strains express multiple pumps that together affect drug efflux (Cannon, et al., 2009). Efflux pump reduces the intracellular azole concentrations below the level at which Erg11p is inhibited causing a significant drug resistance (Cannon, et al., 2009).

There are two major families of efflux pump responsible for drug resistance: 1) the major facilitator superfamily (MFS); and 2) the ATP-binding cassette (ABC) superfamily. MFS pumps efflux a limited range of substrates, whereas ABC transporters have broader specificity and are more clinically significant.
**Efflux pumps:**

Efflux pumps are transport proteins involved in translocation of substrate across a cell membrane against the concentration gradient. The ABC proteins are primary transporters that utilize the hydrolysis of ATP as a source of energy. MFS pumps are secondary transporters that use electrochemical gradient across the plasma membrane (Lamping, et al., 2010). Both transporters contain specific protein domains, including nucleotide binding domains (NBDs) in ABC pumps and transmembrane domains (TMDs) in both ABC and MFS pumps (Cannon, et al., 2009).

There are two subfamilies of MFS transporters involved in drug efflux that differ mainly in the number of transmembrane spans (TMS) within the TMD: 1. DHA1 (drug: H\(^+\) antiporter 1; 12 TMS); and 2) DHA2 (14 TMS) (Sá-Correia, et al., 2009). *S. Cerevisiae* has 12 DHA1 and 10 DHA2 transporters, most of which are involved in multidrug-resistance (MDR). The first characterized MFS transporter gene was CaMDR1 (also named \textit{BEN}) from *C. albicans*. This gene conferred high resistance to benomyl and methotrexate when expressed in *S. cerevisiae* (Cannon, et al., 2009). Overexpression of CaMdr1p in *C. albicans* conferred resistance to fluconazole.

ABC transporters are present in all kingdoms of life. This protein is divided into three major classes. Class I proteins include the transporters whose TMDs and NBDs are fused in a single polypeptide chain. Many of these transporters cause resistance to antimicrobial agents. Class II proteins lack TMDs and are unable to transport substrates. They are, however, able to perform other actions such as translation of mRNA or DNA repair. In Class III transporters, two separate polypeptide chains encode the TMD and NBD dimers. These transporters serve as bacterial importers (Davidson, et al., 2008).
The ABC proteins of *S. cerevisiae* are divided into six subfamilies: 1) PDR (pleiotropic drug resistance), 2) MDR (multidrug resistance); 3) MRP (multidrug resistance-associated protein); 4) RLI (RNase L inhibitor); 5) ALDP (adrenoleukodystrophy protein); and 6) YEF3 (yeast elongation factor EF-3). Of these, the PDR, MDR, and MRP are most often associated with antifungal resistance. The major pumps involved in azole resistance of *C. albicans*, *C. glabrata*, *C. krusei* and *C. neoformans* are PDR’s. CDR1 is a well-studied ABC transporter found in *Candida* species and *Cryptococcus neoformans* involved in resistance to fluconazole and other azoles. This transporter is a member of the PDR family of ABC transporters, which is only found in plants and fungi. Based on gene sequencing, *cdr1* appears to be similar in structure to human P-glycoprotein (Pgp), a multidrug pump associated with resistance to various chemotherapeutic agents (Ghannoum and Rice, 1999).

Phylogenetic analysis suggests that there are 49 ABC proteins in humans, which have been organized into seven sub-families. There are many human ABC transporters responsible for MDR, among which are P-gp, and ABCG2. These human transporters, as well as Cdr1 of *C. albicans* and Cgr1 of *C. glabrata*, are functional homologs of the *S. cerevisiae* Pdr5 transporter (Rutledge, et al., 2011). Alterations in human transporters or in their cellular expression are associated with several diseases including cystic fibrosis, adrenoleukodystrophy and multidrug resistant tumors (Gottesman and Ambudkar, 2001).

**ABC transporters**

ABC transporters are one of the largest classes of membrane proteins. ABC transporters use the binding and hydrolysis of ATP to translocate a diverse number of substrates, ranging from ions to macromolecules, across membranes against a
concentration gradient. They couple transport of a substrate across the membrane to the hydrolysis of the phosphate bond between the $\gamma$- and the $\beta$-phosphate of ATP. The free energy released when ATP is converted into ADP and orthophosphate can be used to transport the substrates across the membrane (Ter Beek, et al., 2014).

ABC transporters are localized in the plasma membrane, and ATP hydrolysis occurs on the cytoplasmic side. In eukaryotes, some ABC transporters are located in organelles membranes and ATP hydrolysis occurs on the cytosolic side of the membrane, except for mitochondria and chloroplast where the ATP-binding domains of the transporters are located on the matrix or stroma side. The membrane side of ATP binding and hydrolysis is termed the cis-side, and the opposite side is called the trans-side (Ter Beek, et al., 2014).

These integral membrane proteins come in two varieties as either importer, bringing nutrients and other molecules into cells (for example E.coli BtuCD protein, a vitamin B12 importer), or as exporters that pump toxins, drugs and lipids across out of cells (Rees, et al., 2009). Members of the ABC transporter family are ubiquitously expressed and are present in all kingdoms of life. While importers are only present in prokaryotes, exporters are present in both prokaryotes and eukaryotes. In E. coli, ABC transporters constitute the largest protein family, including ~80 distinct systems representing 5% of the genome, and in humans ~50 ABC transporters are present. Loss-of-function mutations in ABC transporter genes are commonly associated with several disorders including cystic fibrosis, Tangier disease, hypercholesterolemia and diabetes (Rees, et al., 2009).

**Structure of ABC transporters**
Most ABC transporters are composed of a highly conserved topology of four essential domains, two cytoplasmic NBDs and two TMDs. In eukaryotes, most ABC proteins exist as full transporters with two TMDs and two NBDs, but they are not always arranged in the same order. In a full transporter, the order of TMDs and NBDs in the sequence determines the topology of the protein. In the forward topology, the TMDs precede the NBDs, whereas in the reverse topology the NBDs come first (Rea, 1999/ Rutledge, et al., 2011). A schematic representation of the topology of various ABC transporters is presented in Figure 2. In bacteria, a TMD is fused to a NBD forming a “half-transporter”, which will homodimerize or heterodimerize with another “half-transporter” to form the full functional transporter.

![Figure 2: Topology of ATP-binding cassette (ABC) transporters.](image)

**Figure 2: Topology of ATP-binding cassette (ABC) transporters.** ABC transporters contain four domains, two trans-membrane domains (TMDs) and two nucleotide-binding domains (NBDs). **A.** In the forward topology, the TMDs precede the NBDs. **B.** NBDs come first in the reverse topology.
In most ABC transporters, each TMD consists of six α-helices connected by ICLs and ECLs. The TMD’s α-helices are located in the membrane bilayer and form a transport channel or binding pocket, through which substrates are transported in the membrane bilayer (Locher, 2009). The TMDs of ABC transporter efflux pumps share variable sequences and architectures, reflecting the chemical diversity of the translocated substrates.

In contrast, NBDs are cytosolic and contain highly conserved sequence motifs that function in binding and hydrolysis of ATP and other nucleotides (Locher, 2009). Each NBD consists of two subdomains: a large ATP-binding catalytic domain and a small signaling domain. The catalytic domain has an α/β folding motif and contains well-conserved sequence elements, including the Walker A and B motifs, the D-loop, the C-loop (Signature) and the switch region. The signaling domain is composed of α-helices and contains the Q-loop and in some cases the X motif (Schmitt and Tampe, 2002). Together these structural elements are arranged into two ATP-binding sites in a NBD dimer. Each ATP-binding site is formed from six conserved motifs, four from one NBD and two from the opposite NBD (Figure 3) (Zolernicks, et al., 2007; Davidson et al., 2008). Multiple residues from NBD motifs interact with the ATP molecule to break the high-energy γ-phosphate bond. Residues of the Walker A, Walker B, H-loop and Q-loop of one NBD and the D-loop and Signature motif of the opposite NBD interact with water, magnesium, and ATP for hydrolysis at one ATP-binding site (Davidson, et al., 2008). The function of each motif in ATP binding and hydrolysis is shown in Table 1.
Figure 3: Nucleotide binding motif arrangement of an ABC transporter.

The catalytic domain contains well-conserved sequences such as the Walker A (A) and Walker B (B) motifs, the D-loop (D), and the switch region. The signaling domain contains the Q-loop (Q). The structural elements are arranged into two ATP-binding sites in an NBD dimer.
Table 1: The functions of each nucleotide-binding motif (ter Beek, et al., 2014).

<table>
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<tr>
<th>Motif</th>
<th>Function</th>
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<tr>
<td>Walker A (P-loop) (GXXGXGK(S/T))</td>
<td>Coordinates Mg$^{2+}$ and the β- and γ-phosphates of ATP.</td>
</tr>
<tr>
<td>Walker B (ΦΦΦΦDE, where Φ is a hydrophobic amino acid)</td>
<td>Coordinates Mg$^{2+}$ via the conserved aspartate residue.</td>
</tr>
<tr>
<td>D-loop (SALD)</td>
<td>Stabilizes the NBD: NBD interactions.</td>
</tr>
<tr>
<td>H-loop (Contains a highly conserved histidine residue that forms a hinge between a β strand and an α helix near the C terminus of the NBD)</td>
<td>The conserved His residue interacts with the conserved Asp (D-loop), the Glu residue of the Walker B motif, and with the γ-phosphate of the ATP. It assists with the positioning of the attacking water, ATP, and Mg$^{2+}$.</td>
</tr>
<tr>
<td>Q-loop (Approximately eight residues with a conserved Q residue at its N terminus)</td>
<td>Coordinates the γ-phosphates of ATP and contracts the intracellular loops. Conformational changes in the Q-loop allow the conserved Gln residue to move in and out of the active site during the hydrolysis cycle, forming the active site when Mg-ATP is bound and disrupting it once ATP is hydrolyzed. Part of the signal transmission interface.</td>
</tr>
<tr>
<td>X-loop (TEVGERG)</td>
<td>Interacts with both coupling helices (ICL1 and ICL2) of the TMDs. Part of the signal transmission interface.</td>
</tr>
<tr>
<td>Signature (C-loop) (LSGGQ)</td>
<td>Orient the γ-phosphate of ATP.</td>
</tr>
</tbody>
</table>
Transport cycle of ABC transporters

ABC transporters adopt two different conformations in which the substrate-binding site is accessible from either the cis- or the trans-side. The alternation between two conformations allows substrate translocation across the membrane. Binding of a substrate on one side of the membrane and release on the opposite side is regulated by ATP binding and hydrolysis. Sauna and Ambudkar (2001) proposed a kinetic cycle for ABC efflux pumps using human P-glycoprotein (Pgp) as the transporter of study. This study suggested that when the substrates binding site within the TMDs is in the inward facing conformation the substrate will bind and move from the cytosol or lipid bilayer into the substrate-binding pocket within the TMD. Binding of the substrate into TMD causes a conformational change resulting in a closed dimer, bringing the NBDs into close proximity allowing for ATP binding at the two ATP binding sites. The binding and hydrolysis of ATP at the first ATP binding site allows the TMD to change to an outward facing conformation, which opens toward the external space and effluxes the substrate outside the cell. Binding and hydrolysis of ATP at the second ATP binding site resets the pump to the inward facing conformation to allow the binding of more substrate for the next transport cycle (Sauna and Ambudkar, 2001). This transport cycle is illustrated in Figure 4.
Figure 4: Transport cycle of ABC exporters. The resting state of the transporter is in an inward facing conformation. Substrate enters into the substrate-binding pocket bringing the NBDs into close proximity allowing ATP to bind. ATP binding and hydrolysis induces a conformational change in the TMDs switching from an inward facing conformation to an outward facing conformation allowing for efflux of the substrate (Szöllősia, et al., 2018).

Signaling interface in ABC transporters

Communication between the TMDs and NBDs is necessary to couple energy from ATP hydrolysis with conformational changes in the TMDs for substrate efflux. The communication between TMDs and NBDs is thought to take place through a signaling interface, which is also referred to as the “transmission interface”. High-resolution atomic structure of the bacterial Sav1866 multidrug transporter shows the contact between the ICLs of the TMDs and the NBDs (Locher, et al., 2006). When ADP was bound to the homodimer, it adopted the outward-facing conformation. ICL2, which connects TMH2 and TMH3, makes contact with the opposite NBD at two conserved sequences, the X-loop and the Q-loop, to create a trans conformation. ICL1, however, makes only limited contact with the cis NBD (Dawson and Locher, 2006). Thus, it appears that the signal is transmitted from the TMDs to the Q-loop of the NBD through the ICLs.

Signal transmission causes the Walker A motif to be displaced, which allows ATP to access the nucleotide binding site. The Signature motif from the other NBD aligns with
the rest of the nucleotide-binding pocket and a closed dimer is formed. The formation of a closed dimer (power stroke for transport) results in a conformational change in the TMDs leading to substrate release. ATP hydrolysis at the NBD leads to the formation of ADP and Pi, destabilizing the NBD dimer. Electrostatic repulsion between ADP coordinated with the Walker A motif of one NBD and Pi, with the signature domain of the other NBD, destabilizes the closed NBD dimers. Several studies of human ABC transporter symmetric P-gp, a homolog of Sav1866, used cross-linking between cysteine-modified residues of ICL2 and the trans Q- or X-loops. Results of these studies suggest that P-gp might have a transmission interface similar to Sav1866 and lends credence to the signal pathway suggested by the Sav1866 atomic structure. Taken together, these studies suggest that ABC transporters have a well-conserved transmission interface (Zolnerciks, et al., 2007).

The Pdr5 yeast ABC transporter

ABC transporters confer resistant to different substrates in process known as multidrug resistance (MDR). In yeast, MDR is called pleiotropic drug resistance (PDR). An important member of the PDR subfamily of ABC transporters is pleiotropic drug resistance gene 5 (Pdr5). Pdr5 is the major drug efflux pump of baker’s yeast *Saccharomyces cerevisiae*. It is a founding member of the PDR subfamily of ABC transporters, which is only present in slime molds and fungi. Pdr5 belongs to the ABCG (PDR) subfamily of ABC transporters. The *pdr5* gene was first identified as a DNA sequence that caused hyper resistance to cycloheximide and sulfometuron methyl when it was overexpressed in wild-type (WT) yeast cells. Mutants of the *pdr5* gene were
hypersensitive to these drugs relative to an isogenic WT control bearing only the cloning vector (Leppert, et al., 1990).

The PDR subfamily is clinically significant. Pdr5 is a functional homolog of P-gp, MRP1p and ABCG2, three human ABC transporters involved in MDR against chemotherapeutics. Pdr5 shares 71% sequence identity and 85% sequence similarity with *Candida glabrata* CGR1 multidrug transporter, and 55% sequence identity and 76% sequence similarity with *Candida albicans* CDR1 (Rutledge, et al., 2011). These transporters are overexpressed in multidrug resistant clinical isolates. The high sequence similarity and functional homology of Pdr5 to clinically relevant ABC transporters make it an important model transporter to study the mechanism of MDR (Rutledge, et al., 2011).

Pdr5 is a full-length, 160 kDa plasma membrane protein, consisting of 1511 amino acids in a single polypeptide chain. The PDR subfamily is highly asymmetric, characterized by a reverse orientation relative to the standard arrangement of NBDs and TMDs, and a highly unusual set of short ICLs. The structural model of Pdr5 is illustrated in Figure 5.
Figure 5: Structural model of ABC transporter Pdr5. The predicted topology of Pdr5 containing 12 TMHs and 2 NBDs, organized in reverse order. The two NBDs are shown as blue and red ovals. Helices in TMD1 (519–795) are shown in teal and those in TMD2 (1203–1502) are shown in magenta.

One Pdr5 ATP-binding site is entirely canonical and catalytic; the other is deviant and non-catalytic (Golin and Ambudkar, 2015). The canonical site is made up of the Walker A, Walker B, and Q-loop motifs of NBD2 and the Signature and D-loop motifs of NBD1. The deviant site is constructed from the Walker A-, B-, and Q-loop motifs of NBD1 and the Signature and D-loops of NBD2. The deviant ATP-binding site lacks a catalytic glutamate residue in the Walker B motif and contains deviant sequences in the Walker A, Signature and Q-loop motifs. A schematic diagram of the proposed domain relationships of Pdr5 and its ATP binding site found in Figure 6.
Figure 6: Pdr5 domain relationship and organization of the NBDs into canonical and deviant ATP sites: A. Diagram of Pdr5 organization. NBD1 is followed by TMD1 composed of six $\alpha$-helices followed by NBD2 and TMD2 also composed of six helices. B. Pdr5 is an asymmetric transporter with one canonical ATP site made up of the Walker A and B motifs and Q-loop region from NBD2 and the C- and D-loop from NBD1. The second site is atypical (Walker A and B motifs and Q-loop from NBD1, C- and D-loop from NBD2) and its Walker B motif lacks a catalytic glutamate residue. This pattern of alterations is found only in the Pdr ABC subfamily of fungal transporters. The diagram is from Furman et al. (2013).

In 2013, Furman, et al. proposed a model for the role of the deviant ATP binding site as presented in Figure 7. Binding of ATP at both ATP binding sites in the inward facing conformation causes dimerization of the NBDs, but hydrolysis is limited to the canonical site. Binding and nucleotide exchange at the deviant site allow a change from an inward-facing, drug-binding conformation to an outward-facing, drug-releasing conformation. Residues such as Glu-1013 and Asp-1042 mediate this conformational change. At the canonical site, ATP hydrolysis restores the transporter to a drug-binding
conformation and causes the release of unhydrolyzed ATP. The members of this subfamily have an extremely high basal level of ATPase activity that is not stimulated by the addition of a transport substrate. The pump is always running regardless of whether the substrate is present or not (Furman, et al., 2013).

**Figure 7: Role of the deviant ATP binding site of Pdr5.** ATP is bound at both sites, causing dimerization (II) of the NBDs, but hydrolysis is limited to the canonical one. Binding and nucleotide exchange (black for red) at the deviant site (III), indicated by the green border, allow a change from an inward-facing, drug-binding conformation to an outward-facing, drug-releasing conformation. This is mediated by residues such as Glu-1013 and Asp-1042. ATP hydrolysis at the canonical site (IV) restores the transporter to a drug-binding conformation and causes the release of unhydrolyzed ATP (blue).

High basal ATPase activity is a common feature in the Pdr fungal subfamily (DeCottignies, et al., 1994; Golin, et al., 2007; Ernst, et al., 2008). This activity is allosterically inhibited by a subset of strong transport substrates such as clotrimazole (Golin et al. 2007; Ernst et al.2008). Gupta, et al. suggested that this inhibition prevents the conformational shift from the outward-facing orientation to the inward-facing conformation after the drug is released to the outside of the cell (Gupta, et al., 2011).

Pdr5 possesses high basal ATPase activity (~200 nmol min⁻¹ mg⁻¹), followed by GTPase (~70 nmol min⁻¹ mg⁻¹), UTPase (~35 nmol min⁻¹ mg⁻¹) and CTPase activity. In
2007 Golin, et al. demonstrated that the UTPase and CTPase activities of Pdr5 were low and the $K_m$ values were high, suggesting that UTPase and CTPase activities did not perform a significant physiological role. The GTPase activity, however, had a $K_m$ of ~1 mM and was therefore within physiological range and enzyme activity was relatively high. The GTPase activity was also more resistant to allosteric inhibition by clotrimazole. The low $K_m$ (GTPase) and the significant resistance to clotrimazole trans-inhibition suggests that Pdr5p may use GTP as another source of energy (Golin, et al., 2007).

Furthermore, Golin et al. (2007) demonstrated that the transport of $[^3]$H-chloramphenicol in plasma membrane (PM) vesicles was mediated by Pdr5 GTPase activity. In the absence of either Pdr5 or GTP, no transport was detected.

**Pdr5 substrate specificity**

Pdr1 and Pdr3, a zinc-finger transcription factors that have been previously characterized, transcriptionally regulate Pdr5. (Katzmann, et al., 1994; Golin and Ambudkar, 2015). It has been shown that Pdr5 mediates resistance to a variety of antitumor agents, dyes, small peptides, and hormones. Pdr5 mediates drug resistance to substrates that are structurally and functionally different (Meyers, et al., 1992). Golin et al. (2003) demonstrated that molecular volume, rather than hydrophobicity of the compound, is the most important factor in determining substrate strength. Thus, two compounds with the same size but with substantial differences in log $P$ had similar substrate strength. Strong Pdr5 substrates had surface volumes of 200-300 cubic angstroms. Compounds less than 90 cubic angstroms were poor substrates, which indicated that Pdr5 played little or no role in mediating their transport. Compounds above 300 cubic angstroms showed a gradual decline in substrate transport capability (Golin, et
The same study also demonstrated that Pdr5 employs multiple drug binding sites much like P-gp, which appear to overlap (Hanson, et al., 2005). Therefore, it was proposed that large compounds have more residues to interact with than those with less surface volume, meaning that larger compounds are stronger substrates for Pdr5 than smaller ones (Golin, Ambudkar, and May 2007).

The Pdr5 signaling interface

As is the case with all ABC transporters, Pdr5 must have a communication pathway between the TMDs and the NBDs for substrate transport to occur. Since Pdr5 has one deviant ATP binding site and extremely short ICLs, it was not clear whether its transmission was similar to that of Sav1866 and P-gp’s described above. Studies in our laboratory employing suppressor genetics indicated that Pdr5 uses a similar pathway with one major twist: Pdr5 appears to employ a cis rather than a trans pathway (Sauna, et al., 2008), with interactions between ICL-2 and the Q-loop of NBD1, rather than NBD2.

The first structure of an ABCG subfamily member (ABCG5/ABCG8) was reported (Lee, et al., 2016), and it is clearly in the cis conformation. As Pdr5 is also a member of this asymmetric subfamily, our genetic observation was structurally confirmed. A similar observation was recently made with the ABCG2 multidrug efflux pump (Manolaridis, et al., 2018). Thus, in at least two atomic structures of the mammalian ABCG family members, ICL-2 contacts the amino-terminal Q-loop of NBD1 rather than NBD2, in contrast to members of other ABC subfamilies of eukaryotic efflux pumps where the trans orientation is observed.
Pdr5 as a molecular diode

When a drug molecule is released from the drug-binding pocket, it must be prevented from reentry through the transporter, which is now in an outward drug releasing conformation. The standard model of ABC transporters, which is supported by structural and biochemical studies in bacterial and mammalian systems, demonstrates that ABC transporters oscillate between high-affinity substrate-binding and low-affinity substrate-releasing conformations. The outward drug-releasing conformation was thought to prevent the rebinding of the drugs to the binding sites because of the reduced affinity. Studies of drug binding with the photoaffinity substrate iodoarylazidoprazosin (IAAP) demonstrated a 10×–30× reduction in substrate-binding affinity when P-gp switched to its outward-facing conformation (Sauna and Ambudkar, 2000). Though a decrease in binding affinity of this magnitude would allow drug release, this reduction might not be sufficient to prevent reentry of the substrate through the binding sites if the external concentration of the substrate is high (see Mehla, et al., 2014 for more discussion).

A second mechanism was suggested by the observation that increased substrate concentrations inhibited the ATPase activity of the transporter by causing allosteric inhibition (Golin, et al., 2007). Gupta et al. 2011 suggested that this inhibition blocks ATP hydrolysis and locks the conformation of the transporter in an outward facing conformation. Consequently, conformational switching is prevented. In the case of both P-gp and Pdr5, however, a number of clinically important substrates do not inhibit the ATPase activity at even very high concentrations (Downes, et al., 2013).

Gupta et al. 2011, therefore proposed that drugs that do not cause trans inhibition of ATPase activity could slip back through the transporter. Therefore, another alternative
proposed by Gupta et al. 2011 was that ABC efflux pumps are molecular diodes or unidirectional gates that are remodeled after drug release and ATP hydrolysis so that back transport is eliminated even if some drug is bound to a transport site. All three different mechanisms (loss in affinity, trans-inhibition and the presence of a molecular diode) operate to keep the excluded drug substrate from returning to the cell. Recent work in our laboratory lends credence to the diode hypothesis (Mehla et al. 2014).

The Ser1368Ala mutation

The first evidence for a molecular diode was established with the yeast multidrug transporter Pdr5 and the mammalian Tap antigen transporter (Mehla, et al., 2014 / Grossmann, et al., 2014). Mehla et al. (2014) demonstrated that ser-1368 played a critical role in diode function. The S1368A mutation was constructed as part of a systematic study designed to identify drug-binding residues. Previous studies by Egner et al. (1998) demonstrated that mutations in the residues of TMH11 (then predicted to be TMH10) effect the substrate specificity of Pdr5. These mutations include Ser-1360 and Thr-1364. These results suggested that some of these residues form part of a drug-specific binding site. Using a homologous model of Pdr5 (Rutledge et al, 2011), our lab selected and made alanine substitutions in Phe-1363, Thr-1364, Ser-1366, Ser-1368, Phe-1494 (TMH12), and Phe-1495. These alanine mutation phenotypes were tested with structurally different Pdr5 transport substrates. Some of the mutants had mild, drug-specific phenotypes not found in the WT. For example, the T1364A substitution was 2.5 times more sensitive to climbazole (hydrophobic compound) than the WT and had normal transport kinetics for R6G. The S1368A mutant strain, in contrast, exhibited increased drug hypersensitivity to all tested functionally Pdr5 transport substrates. These drugs vary in structure and degree
in hydrophobicity, with a partition coefficient (logP) range of 1.2 (cerulenin) to 5.4 (clo).

In each substrate, the mutation was \(~5–6\) times more sensitive than the WT (Mehla, et al., 2014).

The S1368A mutant was extremely puzzling. Despite its hypersensitivity to structurally different Pdr5 substrates, all of its biochemical features were WT. S1368A exhibited WT drug binding behavior, WT levels of Pdr5 in purified PM vesicles, and WT ATPase activity and normal allosteric inhibition of its ATPase. However, the S1368A mutant had unusual behavior when different transport and reflux assays with R6G were performed.

S1368A was tested first with the standard R6G assay used by several laboratories (Kolaczkowski, et al., 1996). Thus, de-energized cells were pre-loaded with R6G for 90 min and then untransported R6G was removed prior to adding glucose to initiate R6G transport. Transport was initiated by placing the cells in a buffer with glucose, but no R6G. The retained fluorescence was measured at various intervals in mutants and WT cells. Under these conditions, transport took place in the direction of the R6G concentration gradient, the S1368A mutant exhibited a nearly WT phenotype. When such an assay is performed, the R6G in the cells is immediately diluted in a large volume of buffer. The chance that the drug will reflux back into the cell is very low. As a result, S1368A transport was largely unaffected.

In a second series of experiments, a transport experiment was designed so that transport would take place against a concentration gradient of 20\(\mu\)M R6G. To do this assay, cells were placed in a Hepes glucose buffer containing the substrate and the amount of fluorescence remaining in the cells was determined after a fixed-time interval
(90 min). Under these conditions where reflux was likely because the extracellular concentration remained high, the S1368A mutant retained about ten-times more fluorescence than the WT. This was the first suggestion that S1368A created a “leak” in the diode.

In order to directly confirm that the S1368A showed significantly more drug reflux than the WT, cells were loaded with 20 µM R6G for 90 min under de-energized conditions. Following this, the cells were pelleted and resuspended in 20 µM [3H]-R6G in glucose buffer for 15 or 90 min before pelleting the cells and washing away unincorporated isotope. The amount of retained [3H]- R6G was determined. At 15 min, the S1368A mutant had accumulated 3- times as much [3H] R6G as the WT. At 90 min, there was no increase in the WT accumulation, but the S1368A cells continued to incorporate [3H] R6G, and the differential between strains rose to ~6-times. Thus, the transport experiments strongly supported the idea that Ser-1368 plays a direct role in the exclusion of drug reflux during transport. This idea is illustrated in Figure 8. When the external concentration of transported drug is relatively low, reflux is minimized (Figure 8 A). The phenotype is more severe when the extracellular concentration is high, forcing Pdr5 to work against a gradient, the S1368A diode would leak to a much greater extent (Figure 8 B). The S1368A mutant with its leaky gate therefore accumulates a several fold higher concentrations of cytotoxic drugs and exhibits broad, profound hypersensitivity (Mehla, et al., 2014; Golin and Ambudkar, 2015). An important study with the Tap transporter demonstrated a gating function that required the D-loop and the same signal transmission pathway used to transport processed antigen (Grossmann, et al., 2014).
Figure 8: **Pdr5 as a molecular diode.** In the model, blue circles represent transport substrates and blue arrows show the direction of drug passage. Unhydrolyzed ATP is shown in red, and ADP is shown in green. The nucleotide exchange at the deviant ATP site of Pdr5 induces a conformational switch from an inward (drug-binding) to an outward (drug-releasing) conformation. ATP hydrolysis at the canonical site allows the drug to be released and causes remodeling of the transporter to preclude re-entry of the unwanted drug as the transporter is restored to its inward-facing conformation. When the transport is in the direction of the gradient (A) and the external concentration of the drug is relatively low, reflux because of the S1368A mutant phenotype is limited. In (B), the external drug concentration is high, transport is against a gradient and the S1368A mutant phenotype is most severe. Adapted from Mehla et al 2014.

**Bioinformatics analysis of Ser 1368**

Recently, structures of several ABC transporters allowed investigators to see the gate or “plug” that had been inferred from the behavior of the Pdr5 and Tap mutants. Of interest was the relationship between Ser1368 and the residues making up the molecular plug of ABCG2 visualized in the cryo-EM study: Leu554 and Leu 555 (Manolaridis, et al., 2018). An alignment of Pdr5 with ABCG2 demonstrated that Ser-1368 is very close to these residues Leu 554 and Leu555 correspond to Val 1372 and Met 1373 (J. Golin, unpublished observation). An extensive analysis of the Pdr family established that although Ser1368 is unconserved, Val 1372 and Met 1373 are highly conserved (Lamping, et al., 2010).
Ser-1368 lies at the end of TMH 11, several residues away from EL 6. When the sequences of Pdr5 and ABCG2 are aligned, Ser-1368 is quite close to Leu-554 and Leu-555. The corresponding residues in Pdr5 are Val-1372 and Met-1373 (Figure 9A). An extensive bioinformatics analysis of the Pdr subfamily (Lamping, et al., 2010) demonstrates that amino acids in this vicinity are highly conserved (Figure 9B). These include Phe-1369, Gly-1371, Val-1372, and Met-1373. The latter two residues are the first two residues in EL 6. A similar set of residues is found in the amino terminus of Pdr5-extend from Tyr-680 to Ile-685 (Figure 9C). The human ABCG8 half-transporter sequence aligns with the amino rather than the carboxyl set of residues (Figure 9D).
Figure 9: Conservation of residues in the vicinity of Ser-1368.

A. An alignment of Pdr5 with the human half-transporter ABCG2 was performed using the ExPASy bioinformatics database. B. A bioinformatics analysis of 263 Pdr subfamily members by Lamping et al. 2010 was used to evaluate the conservation of the residues that are adjacent to Ser-1368. An X indicates a non-conserved residue. C. The amino acids in the amino-terminal half of Pdr5 corresponding to Ser-1368 are indicated in a similar manner. D. When the ExPASy alignment software is used, the human ABCG8 transporter aligns with the sequence in panel C.

Statement of problem

Sequence alignment of Pdr5 with other Pdr homologs revealed highly conserved residues adjacent to Ser-1368. These appear to constitute a motif (FCGVM, residues 1369-1373) toward the carboxyl-terminus. A bioinformatics analysis by Lamping et al. 2010 showed that Phe-1379 and Gly-1371 as well as Val-1372 and Met-1373 were highly conserved. Furthermore, when the ABCG2 sequence was aligned with Pdr5, Leu-554 and Leu-555 corresponded to Val-1372 and Met-1373. Based on the alignment Val-1372 and Met-1373 may be the actual gate (Figure 9).

While a good deal is known about the biochemistry of ABC transporters, including the properties of their ATPase activity, drug binding, and signaling between the
ATP-hydrolysis and drug binding sites, the requirement for a gating function in a multidrug efflux pump is a relatively recent concept. Aside from the properties of the S1368A mutant and the identification of the diode residues in ABCG2, little is known about which amino acids are essential. It is also not clear whether gating is strongly dependent on the chemistry of the compounds being transported out of the cell. The first goal of this study is to make mutations in conserved residues and use a series of biochemical and cellular assays to determine whether the conserved set of amino acids spanning residues 1369-1373 in the carboxyl-terminus motif of Pdr5 make up a molecular diode motif. A set of corresponding residues in the amino terminal sequence (SMYTGF, residues 678-683) also exhibited conservation (Lamping, et al., 2010). It is important to determine whether the molecular diode is made up of residues from both halves of Pdr5. When the two halves of Pdr5 are aligned, we found that Met-679 is equivalent to Ser-1368 and Gly-682 is equivalent to Gly-1371. The tyrosine, glycine, and phenylalanine appeared highly conserved. The second goal of this study is to investigate whether the molecular diode is composed of residues from both halves of this large, polytopic protein.

This dissertation seeks to fill critical knowledge gaps in our understanding of how the Pdr5 molecular diode operates and could lead to drugs that target the gating mechanism and disrupt its function. This is the first functional analysis of a set of amino acids thought to play a gating role in an ABC transporter. Characterization of mutants in these two motifs might lead to specific chemical inhibitors of these transporters during disease treatment.
Materials and Methods

Yeast strains

All yeast strains used in this study were isogenic and derived from R-1, a strain lacking all plasma membrane ABC transporters including Pdr5 (Δpdr5). In R-1 the PDR5 coding region was replaced with a resistance cassette (KANMX4) and possesses a PDR1-3 mutation. PDR1 is the transcriptional regulator of PDR5. The PDR1-3 mutation causes a 10-fold increased expression of PDR5 and results in Pdr5 transport substrate overexpression (Meyers, et al., 1992). To create the remaining stocks, we transformed R-1 with either the wild type plasmid pSS607 (JG2015) or one containing a site directed Pdr5 mutation described below. The integrated plasmid is under the control of a PDR5 promoter and contains all of the required regulatory sequences for PDR5 expression in yeast. This plasmid contains the yeast URA3 selectable marker and the ampicillin resistance (AMPR) gene for maintenance in E. coli.

Media

All nutrient media were purchased from Research Products International (Mt. Prospect, IL), prepared with MilliQ (MQ) water and autoclave sterilized at 121°C and 15 psi for 30 min. Yeast strains were maintained on yeast peptone dextrose (YPD) medium containing 2% (20g/1 L) dextrose, 2% (20 g/1 L) peptone, and 1% (10 g/1 L) yeast extract. Yeast transformants were selected on synthetic dextrose with histidine (SD+his) media prepared with of 2% (20 g/1 L) dextrose, 0.67% (7 g/1L) yeast nitrogen base without amino acids, and 0.01% (0.1 g/1 L) amino acids and nucleotides as required. Solid YPD and SD media also contained 2% (20 g/1 L) bacto-agar. Bacterial strains were
grown on Lauria-Bertani (LB) medium for plasmid multiplication. LB broth medium was prepared by adding 10 g tryptone, 5 g yeast extract and 10 g sodium chloride in 1 liter of water. To prepare solid medium, 20 g agar was added per one liter of LB broth.

Drug/selective medium was prepared by adding the required chemicals to the various media listed above. After sterilization, the medium was cooled to 55 °C before addition of the appropriate drug. Ampicillin (Amp) was dissolved in water to prepare a concentration of 50 mg/ml and 50 μg/ml was added to LB agar. Geneticin-418 (G-418) was added to the YPD medium at a concentration of 0.2 g/L. 5-FOA (5-fluoroorotic acid) medium was prepared by adding 5-FOA at a concentration of 1.0 g/L to sterilized and cooled SD medium containing 0.1 g/L histidine and uracil. All yeast strains were cultured at 30°C and E.coli was cultured at 37 °C.

Chemicals and drugs

Clotrimazole (clo), cycloheximide (cyh), cerulenin, climbazole, 5-Fluoroorotic acid (5-FOA) and rhodamine 6G (R6G) were purchased from Sigma-Aldrich (St. Louis, MO) and G418 (geneticin) was purchased from Research Products International (RPI) Corp. (Mt.Prospect, IL). Ampicillin was purchased from Fisher Scientific (Waltham, MA). Cycloheximide was dissolved in water. All other compounds were dissolved in dimethyl sulfoxide (DMSO).
Site-directed mutagenesis

Site-specific mutations were introduced into the plasmid pSS607 using Agilent QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA). The plasmid is illustrated in figure 10. It is a shuttle vector suitable for use in *E. coli* and yeast. Although it has an origin of replication for *E. coli* and replicates autonomously in these cells, it has no origin of replication for *Saccharomyces* and must integrate into the chromosomal locator of *PDR5* to survive. Primers were designed using QuikChange Primer Design Program at www.agilent.com/genomics/qcpd. Designed primers were supplied by International DNA Technologies.

Figure 10: Site-directed mutagenesis plasmid pSS607 for Pdr5 mutation. PSS607 plasmid has an ampicillin resistance gene and *E. coli* origin of replication. It also has a wild type *PDR5* gene capable of being transcribed and translated in R-1 yeast strain. The *URA3* gene is used for selection during yeast transformation experiment.
**Polymerase chain reaction (PCR)**

The Quick Change cycling parameters used to make site directed mutations are found in Table 2.

**Table 2: Reaction mixture for site-directed mutations.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>125 ng in 1μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>125 ng in 1μl</td>
</tr>
<tr>
<td>dNTP</td>
<td>1 μl</td>
</tr>
<tr>
<td>Quick solution</td>
<td>3 μl</td>
</tr>
<tr>
<td>DNA Taq polymerase</td>
<td>1 μl</td>
</tr>
<tr>
<td>Template plasmid- pSS607</td>
<td>100-200 ng in 1μl</td>
</tr>
</tbody>
</table>

The total volume was made up to 50 μl with sterile RNase free water. Reactions were carried out in a TECHNE TC-3000 thermocycler (Bibby Scientific Ltd, Staffordshire, UK) with the following protocol shown in table 3:

**Table 3: Quick change PCR cycling parameters.**
<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>1</td>
<td>95 °C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Annealing</td>
<td>18</td>
<td>95 °C</td>
<td>20 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 °C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>8 minutes</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1</td>
<td>68 °C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

After the PCR reaction was completed, the reaction mixture was treated with 2.0 µL of DpnI restriction enzyme (100U/ µl) (Agilent-Stratagene Technology, Santa Clara, CA) at 37°C to remove methylated parental plasmid. The remaining mixture was used to transform XL10-Gold ultracompetent *E. coli* cells (see protocol below).
Bacterial transformation

Transformation was performed with XL10-Gold ultracompetent cells. They were thawed on ice and 45 μL of the cells were added to pre-chilled Falcon 4059 tubes for each transformant. 2 μL of β-mercaptoethanol (Stratagene, LaJolla, CA) was added to each tube, swirled to mix, and incubated on ice for 2-10 minutes. 2 μL of DpnI treated DNA was added to the sample tube, 1 μL of 0.01 ng/μl pUC18 was added as positive control plasmid to one reaction tube. There was also a reaction performed without plasmid DNA that served as the negative control. The tubes were swirled to mix and incubated on ice for 30 minutes. The reactions were heat-pulsed in a 42°C water bath for 30 seconds, and returned to ice for 2 minutes. Five hundred μL of pre-warmed LB broth was added to each sample and the tubes were incubated in a 37°C incubator with shaking for 1 hour. Cells were then plated on LB plate media containing 50 μg/mL ampicillin and incubated for 24 hours at 37°C to select for transformants.

DNA extraction from bacterial cells

Plasmid pSS607 was isolated from E. coli using an IBI High-Speed Plasmid Mini Kit (IBI scientific, Peosta, IA). 10 ml LB with 50 μg/ml ampicillin broth cultures were inoculated with the transformants of interest. The cultures were grown overnight at 37 °C with shaking. The cells were pelleted by centrifugation at top speed in a standard clinical centrifuge for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 μL PD1 buffer (RNase A) and transferred to a sterile 1.7 mL microcentrifuge tube. 200 μl of PD2 buffer (SDS and NaOH) was added and the solution was mixed by inversion 10 times. The cells were allowed to lyse for 2 minutes at room temperature. 300 μl of PD3 Buffer (potassium acetate and acetic acid) was added and mixed by inversion.
ten times. The tube was then centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a PD column placed in a 2 mL collection tube and centrifuged for 30 seconds. The flow through was discarded and the PD column placed back into the collection tube. 400 μl of W1 wash buffer was then added to the column and centrifuged at 14,000 rpm for 30 seconds, the flow-through was discarded and the PD column placed back into the collection tube. 600 μl of Wash Buffer containing ethanol was added to the column and the tube was centrifuged at 14,000 rpm for 30 seconds. The flow through was discarded and the PD column placed back into the collection tube. The tube was then centrifuged for 3 minutes at 14,000 rpm to dry the PD column. The dried PD column was then placed into a 1.5 ml microcentrifuge tube and 50 μl of elution buffer added to the column.

The column was allowed to stand at room temperature for 2 minutes and then centrifuged for 2 minutes at 14,000 rpm to elute the DNA. The eluted DNA was then transferred to a labeled microcentrifuge tube and the DNA concentration and purity was measured using a Thermo Scientific Nanodrop 2000 spectrophotometer and analysis software (Thermo Fisher Scientific Inc. Wilmington, DE).

**DNA sequencing**

Plasmid DNA extracted from bacteria transformants was sent to Retrogen (SanDiego, CA) for sequencing. The nucleotide sequences were translated into amino acid sequences by ExPASy translation tool (http://web.expasy.org/translate/). The resulting sequence was used in a *S. cerevisiae* WU-BLAST2 search (http://www.yeastgenome.org/cgi-bin/blast-sgd.pl) to compare the results of sequencing to the canonical PDR5 sequence.
Yeast transformation

The R-1 *S. cerevisiae* strain was transformed with the mutant plasmid DNAs by the lithium acetate method using a Sigma-Aldrich kit and protocol (St. Louis, MO). Twenty mL of YPD broth was inoculated with R-1 cells from YPD plate media and incubated at 30 °C with shaking (200-250 rpm) until the culture reached stationary growth phase. Cells from this culture were diluted into 100 mL of YPD broth medium in a 500 mL sterile flask until an A600 of ~0.3 was reached. The culture was incubated at 30°C with shaking for 5-6 hours until an A600 of >0.8 and <1.5 was reached. The cells were then collected after centrifugation at 5,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was washed by resuspending in 50 ml sterile water and centrifuged again at 5,000 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in lithium acetate transformation buffer (supplied with kit) to make cells competent for transformation. The competent cells were immediately transformed.

The required number of sterile microfuge tubes were set up; one for each transformation and one each for a positive and negative control. 10 μl (10 mg/ml) of single stranded salmon testes DNA was added into each sterile microfuge tube. 100-200 ng of plasmid DNA was added to microcentrifuge tubes containing single stranded salmon testes DNA. The mixture was vortexed (no plasmid DNA was added for the negative control). Commercially prepared plasmid was used as a positive control. 100 μl of competent cells in transformation buffer was added to each microfuge tube and vortexed. 600 μl of polyethylene glycol/lithium acetate/tris/ EDTA (PLATE) buffer was added to the reaction mixture and vortexed. The tubes were incubated at 30 °C for 30 min...
with shaking. The cells were then heat shocked at 42 °C for 15 min in a heat block. Tubes were centrifuged at 16,000 x g for 3 seconds to sediment cells. The supernatant was removed and replaced by 500 µL of sterile milliQ water. 100 µl of the cell suspension was plated on SD+his plates and incubated for up to 3-4 days at 30 °C to allow transformants to grow.

**Verification of proper plasmid integration**

5 FOA testing was performed to determine if PDR5 integrated properly within the R-1 strain by homologous recombination. The experimental scheme using 5- FOA illustrated in Figure 10 was utilized. The pSS607 plasmid has *URA3* gene, which was used as a selectable marker to select yeast transformants by plating cells on uracil-lacking medium. The pSS607 plasmid has the entire *PDR5* gene including upstream, coding and downstream regions where R-1 strain contains *PDR5* upstream and downstream regions but the entire coding region of *PDR5* was removed and replaced with a kanamycin (G418) resistance gene.

In the schematic diagram shown below (Figure 11), homologous regions between the plasmid and the chromosome undergo crossing over (homologous recombination) to reverse the initial integration event. Colonies arising on media containing 5-FOA would undergo one of two potential recombination events resulting in the loss of *URA3* and either *PDR5* or *KAMNX4*. Cells that have lost both *URA3* and *KAMNX4* and retained PDR5 were retained for further study. This event can be recovered by plating cells on 5-FOA medium. Cells containing a functional *URA3* gene die on this medium. Cells that have lost this gene survive.
To select 5-FOA recombinants, yeast transformants were inoculated into 2 mL YPD broth and grown overnight at 30°C with shaking. Cells were pelleted by centrifugation at 6,000 x g and the supernatant was discarded. The cells were resuspended in sterile water to wash away the residual medium and pelleted using centrifugation at 6,000 x g. The supernatant was discarded, and the cell pellet resuspended in 1 mL of sterile water. A 1:100 dilution of cells was then prepared using 10 µL of cell suspension and 990 µL of sterile water. Aliquots of 50 µL of sample were inoculated and spread across 5-FOA medium and incubated at 30°C for 72 hours to obtain growth of stable transformants containing the PDR5 gene. These transformants were then streaked onto both YPD and YPD containing Kanamycin (G418). The plates were incubated at 30°C for 48 hours.
Figure 11: Experimental strategy for transformant selection. Colonies in which the cells successfully integrated PDR5 are selected on media containing 5-FOA. Colonies arising on media containing 5-FOA must have undergone one of two potential recombination events resulting in the loss of URA3 and either PDR5 or KAMNX4. Cells that lost both URA3 and KAMNX4 and retained PDR5 are retained for further study.

**Determination of relative drug resistance for Pdr5 transport substrates**

To test for drug resistance, a volume of overnight culture containing $0.5 \times 10^5$ yeast cells was added to 2 ml sterile YPD broth containing the drug concentrations to be tested. The tubes were incubated at $30^\circ C$ for 24 hours while shaking. Following this, the final cell concentration in the tubes was measured by determining the absorbance at 600 nm. For each strain, an untreated culture served as growth control. The cell growth in the untreated control was set as 100%. The percentage growth in the drug treated cultures as a function of drug concentration using GraphPad Prism 8 software (San Diego, CA).
Whole cell R6G assay Against a concentration gradient

To test the transport ability of WT or mutant strains to transport rhodamine 6G (R6G) in whole cells against an R6G concentration gradient, a fluorescence-activated cell-sorting (FACS) assay was used. Cells were grown overnight in YPD medium to a concentration of ~ 10^7 cells/ml at 30°C. The cells were pelleted by centrifugation at 14,000 rpm and washed with MQ water. The cells were then preloaded at 30 °C with 10 μM R6G in 500 μl N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes) buffer (0.02M, pH 7.0) plus 1 mM glucose and 10 μM R6G for 90 min. Next, the cells were pelleted 14,000 rpm at 4°C in a microcentrifuge and the R6G-containing buffer was removed. Following this, the cells were washed with cold HEPES minus glucose and resuspended in 500 μL Hepes minus glucose. The fluorescence of R6G was measured using a Becton-Dickinson FACS (Franklin Lakes, NJ). The excitation wavelength was 488 nm and emission wavelength were 585 nm. The resulting data were analyzed using a Cell Quest program (BD Biosciences, San Jose, CA). The retained fluorescence was expressed in arbitrary units (a.u).

R6G transport in the direction of concentration gradient

R6G transport in the direction of a concentration gradient was also investigated. To do this, 0.5 × 10^7 cells of mutant or WT cells were pre-loaded with 10 μM R6G under energy-depleting conditions for 90 min at 30˚ C. Following this, the excess R6G was removed by centrifugation at 14,000 rpm. Next, the cells were washed once with 500 μl Hepes buffer and pelleted in a microcentrifuge tube. Following this, cells were suspended in Hepes glucose buffer (pH 7.0) without R6G to initiate R6G efflux. The amount of remaining fluorescence in cells were determined at 5, 7.5, and 10 minutes with a
fluorescence activated cell sorter after placing the tubes in ice water bath to stop transport.

[^3H]-R6G reflux assay

To perform experiments with [3H] R6G that measured reflux during transport, 0.5 × 10^7 cells of various strains were loaded with 20 μM nonradioactive R6G made up in 0.02 M Hepes buffer (pH 7.0) without glucose in a final volume of 500 μl for 90 min at 30 °C. Next, the cells were washed with Hepes minus glucose buffer and resuspended in 500 μl of YPD medium containing 20 μM [3H] R6G. The cells were incubated at 30 °C for 15 min. Transport was stopped by placing the assay tubes in an ice water bath. The cells were pelleted 14,000 rpm at 4°C in a microcentrifuge cells and the supernatants were discarded. The pellets were washed three times with 1 ml of cold YPD medium before resuspending in 100 μl of this medium and counting β-ray emission was counted in a Triathler liquid scintillation counter (LabLogic).

Purification of plasma membrane vesicles from yeast cells

Purified plasma membrane vesicles were prepared by Dr. Hadiar Rahman using the following protocol (Adopted from L Serrano and A. Goffeau modified by Ernst, et al., 2008). Two days prior to performing the isolation of PM vesicles, 50 ml YPD broth was inoculated with the desired yeast strain and grown overnight at 30°C with shaking. Add 80 mL sterile 20% glucose into each 2 L flask containing 720 mL YP media. Approximately 15 hours prior to harvest, 750 ml YP broth was inoculated with Final OD600= 0.05x10^7 cells from the initial starter culture and incubated at 25°C with shaking 140 rpm.
Before harvesting at 15-16 hours, the absorbance at 600 nm of each culture was measured to make sure that the cultures were in the exponential phase. Then, 80 mL Boost Media containing Yeast Extract and Peptone was added per flask and incubated at 200 rpm and 25°C. After 5 hours, the cell growth was measured at OD 600. If OD600 was 3.5, the cells were then harvested by centrifugation at 5000xg for 15 minutes at 4°C in a Sorval centrifuge using the GS-3 rotor. Cells were washed with 15 mL MQ per 800 ml culture flask and transferred to a 50 mL falcon tube at 4°C.

The pellets were resuspended in MQ water and filled up to 84 ml in a 100 ml cylinder. Then, the resuspended pellets were transferred into pre-chilled 250ml Glass Beaker. The beaker was stirred by hand, and 5 ml 1 M Tris-acetate pH 7.5 and 1 ml 0.5 EDTA pH 8.0 were added.

270 ml of ice-cold glass beads and 2 protease inhibitor tablets (Biospec, Bartlesville, OK) were added to a bead beater container. The solution with cells was then transferred to the container, and the container was filled with MQ water. The beater was cooled in an ice bath the entire time to avoid heat production during stirring. The bead beater was set on mode of 5 x 1 min. There was a 1 min break between every stirring cycle. After the disruption, the homogenate was filtered with a glass filter to remove the glass beads. The lysates were transferred to a pre-chilled 250ml beaker and filtered through a 60-75 ml filter funnel. The beads were washed two times with 70 ml TAEG buffer. Centrifugation was performed with the JA 10 rotor (average radius rav = 12 cm) at 2600 rpm to remove non-disrupted cells, and nuclei.

The supernatant from centrifugation was centrifuged with the JA 25.50 rotor (average radius rav = 8.2 cm) for 40 min 20000 xg, 4°C. At the same time, one protease
inhibitor tablet was added to 50 ml TAE buffer. The solution was needed for the
resuspension of the plasma membrane. The supernatant was then discarded; the pellet
(mostly mitochondria and plasma membrane vesicle) was resuspended in 12 ml TAE
buffer with protease inhibitor. The centrifuge tubes were washed with 4 ml TAE buffer
containing a protease inhibitor. Both parts of the solution were collected and combined in
a 50 ml falcon tube.

The protein concentration of the PM vesicles was determined with the
CoomassiePlus assay according to the manual (Thermo Scientific, Rockford, Il). The
protein concentration was tested three times to ensure that it is in the range of the
standard, 800 μl of a 1:40 dilution (40 μl into 1580 μl TAE). The TAE buffer was used as
the background. For this assay, 50 μl of the sample were mixed with 1500 μl of the
Coomassie Plus reagent and incubated for 10 min. MQ water was used as blank and then
the samples were scored at OD595.

The plasma membrane vesicles were stirred in a prechilled 125/150ml glass
beaker in cold room (at 4 °C), and vesicles were adjusted to a protein concentration of
5mg/ml with (5-7 ml) of 1 M sodium acetate. The pH of the solution was adjusted to pH
5.2. The precipitating mitochondria were centrifuged (rotor JA 25.50) for 5 min 7000 xg,
4 °C. The supernatant was then transferred to a prechilled 125/150ml glass beaker with
magnetic stir and was adjusted to pH 7.5 with 2.5 M Tris-acetate pH 7.5.

The purified plasma membrane vesicles were centrifuged with the JA 25.50 rotor
for 30 min 26500 xg, 4 °C. Simultaneously, one protease inhibitor tablet was added to 50
ml 50 mM HEPES (pH 7.0) and stored on ice. The supernatant was discarded, and the
pellet was resuspended in 2 ml 50 mM HEPES (pH 7.0) with protease inhibitor. The
protein concentration was determined with a 1:10 (40 μl) and a 1:4 (50 μl) dilution of the plasma membrane vesicles with 50mM HEPES. After that, the protein concentration was adjusted to 1 mg/ml with 50 mM HEPES (pH 7.0) with protease inhibitor and then split into aliquots of 1 ml with 1.5 ml eppendorf tubes, flash frozen in liquid nitrogen, and stored at -80 °C.

The protein concentration from purified plasma membranes was determined using a bicinchoninic acid (BCA)/ copper reduction protein determination kit (Thermo Scientific, Rockford, Il). Standard curves were created to determine the protein concentration in the samples. Seven 13x100 mm glass tubes were labeled A through G to generate a standard curve. Each tube contained various amounts (0-200 μg) of bovine serum albumin (BSA) as the standard (Thermo Scientific, Rockford, IL). The volume was made up to 100 μl by adding sterile milliQ water.

Additional tubes were labeled in triplicate to test protein concentration in purified PM preparations. Ten μL of sample membrane preparations and 90 μL of milliQ water were added to make up the final volume of 100 μl. For color development, a mixture of 49 parts of reagent A and 1-part reagent B was prepared and 2 ml was added to each of the standard and PM sample tubes under study. All tubes were incubated in a water bath for 30 minutes at 37 °C. The contents of each reaction tube were then transferred to a cuvette and absorbance at 562 nm was measured to determine the protein concentration of each tube. Absorbance at 562 nm versus BSA 2 mg/ml was plotted on the graph using GraphPad Prism software to generate a standard curve. The standard curve was then used to determine the protein concentration of each PM preparation.
**Protein electrophoresis**

SDS-PAGE gel was performed to ensure the presence of Pdr5 protein in the purified PM preparation. Membrane proteins (15-30 µg) were denatured using 5x sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading sample buffer (E enzyme, Gaithersburg, MD) at 37°C for 30 minutes to denature and solubilize the protein. 10 µl of molecular weight standard was loaded into the first lane of the precast NuPage 7% Tris acetate gel (Life Technologies, Frederick, MD) followed by each sample in a separate lane.

SDS buffer (1X) was prepared by diluting 20X stock solution of the running buffer. Proteins were separated by electrophoresis at 150 V in Novex XCell SureLock Mini-Cell electrophoresis chambers. Tris acetate gels were stained in colloidal blue stain (Invitrogen, Carlsbad, CA) for 1-2 hours at RT with gentle shaking, and then rinsed overnight in RO water. On the following day, the gel was scanned for relative concentration of Pdr5 (160 kDa), against control strains and Pma1p (100 kDa). Pdr5p bands in mutant strains were compared to the wild type Pdr5 bands and the gel was used for a western blot.

**Western blotting of PM vesicle proteins**

Western blotting was conducted with 10 µg PM vesicle protein as previously described (Downes, et al., 2013). The transfer from the gel to the nitrocellulose membrane (400 mAmp, 60 min) was performed with an X Cell II minicell apparatus (Invitrogen). Antibodies were purchased from Santa Cruz Biotechnology. The polyclonal goat anti-Pdr5 (yC18) and anti-Pma1 (yN-20) antibodies were diluted 1:1000 and 1:250
respectively. The nitrocellulose membranes were blocked for 30 min with 5% nonfat milk in PBS containing 1% Tween 20. Following this, the filters were incubated with both the Pdr5 and Pma1 primary antibodies overnight at 4°C. The filters were washed three times for 15 min before adding a 1:5000 dilution of secondary antibody (donkey, antigoat IgG horseradish peroxidase; SC2033) and incubating at room temperature for 2 h. Blots were developed with a Novex ECL horseradish peroxidase chemiluminescent substrate reagent kit (Thermofisher). The relative amount of Pdr5 protein was compared with the Pdr5/Pma1 ratio as previously described (Downes, et al., 2013).

**Pdr5 ATPase assay for purified plasma membranes**

ATPase activity was assayed as a function of ATP concentration in purified PM vesicles prepared from mutant strains, isogenic WT (JG2015) and ΔPdr5 controls using a method described by Golin et al. (2007). ATPase activity was tested using a colorimetric reaction, by determining the liberation of inorganic phosphate (Pi) that is released upon ATP hydrolysis. The assays were performed using 13 x 100 mm glass tubes filled with a final volume of 100 μL. 12 μg of plasma membrane and 50μl assay buffer (100 mM MOPS (pH 7.0), 50 mM KCl, 5 mM NaNO3, 2 mM EGTA (pH7.0), 2 mM DTT and 10 mM MgCl2) were added to a 13 X 100 mm glass tube. The volume was made up to 97 μl with sterile reverse osmosis water. 3 μL of ATP was then added to each tube at 15 second intervals to begin the ATP hydrolysis reaction. Reactions were allowed to progress for eight minutes 35°C and were then stopped by adding 100 μL of 5% SDS. The amount of liberated Pi was measured spectroscopically at A880 and the ATPase activity (nmol/min/mg) of the sample was calculated. Kinetic analyses were performed with GraphPad Prism software (SanDiego, CA).
Allosteric inhibition of the ATPase activity by clotrimazole

In order to determine the ability of clotrimazole to inhibit ATPase activity of mutant Pdr5 membrane transporters, ATPase assays were performed as described above, except that a fixed concentration (3 μM) of ATP was used. Clotrimazole was added to test the reaction mixtures. A specific concentration of clotrimazole was added to each tube (0-15 μM) before initiating the

**Statistical data analysis:**

Statistical analyses were performed with Prism Graphpad software. The error bars represent the mean with standard deviation. IC50 assays and clotrimazole inhibition of ATPase activity were analyzed using a two-way ANOVA equation. Differences in expression shown in Western blots were compared using T-test analysis. Difference in R6G reflux and efflux were analyzed using a two-way ANOVA and T-test analysis.

**Bioinformatics analyses**

Alignments between ABC transporters were made with the ExPASy database

(https://www.expasy.org/
Results

Suppressor genetics uncovers two potential diode residues, Ser-678 and Leu-1367

Prior to this study, S1368A was the only identified Pdr5 diode residue. It is located in TMH11. Several related strategies were performed during this study to identify additional diode residues that make up the Pdr5 molecular diode. Our first approach was to look for suppressor mutations of the diode-defective S1368A mutant. We isolated suppressors of S1368A cerulenin hypersensitivity by plating ten independently grown cultures of the mutant on solid medium containing a 4 μM concentration of the drug. Resistant colonies arose with a relatively high frequency of about $1/10^5$. We sequenced the chromosomal DNA from seven independent suppressor mutations (Table 4). Of interest was the S678Y mutant located in TMH5. Ser-678 is adjacent to the highly conserved residues located in the amino-terminal half of Pdr5 that are equivalent to Ser1368-Met1373. Ser-678 is therefore equivalent to Leu-1367 when the amino and carboxyl halves of Pdr5 are aligned. Two S1367F suppressor mutations were recovered along with an A1368V reversion and a P596L alteration in intracellular-loop 1.
To determine whether Ser-678 and Leu-1367 might be part of the molecular diode, we constructed the single S678Y and L1367F mutants and tested their drug resistance to cerulenin, clotrimazole, climbazole, cycloheximide, and tamoxifen relative to the WT control. The properties of these transport substrates are found in Table 5. These Pdr5 transport substrates are structurally and mechanistically distinct. A list of properties provided in Table 5 demonstrates the diverse chemical nature of these compounds. The compounds used comprise both aromatic and non-aromatic characteristic. The range in molecular volume is significant (197-356 cm$^3$) as is the compounds hydrophobicity, characterized as its solubility in octanol versus water ratio (logP). These values range from 0.9-7.9. The phenotypes of both mutants were drug specific. Both mutants exhibited hypersensitivity to clotrimazole (Figure 12A) and had IC$_{50}$ values ($\sim$ 5.0 µM) that were about half the WT ($\sim$ 12 µM). A two-way ANOVA test indicated that the mutant curves were significantly different from the WT. The modest hypersensitivity of the S678Y mutant to tamoxifen (Figure 12B) was also significant.
Both mutants were significantly more resistant than the WT to climbazole (Figure 12C) and to cerulenin, the agent used to select the suppressors (Figure 12D). In contrast to the S678Y mutant, the L1367F strain was also hyperresistant to cycloheximide (Figure 12E) relative to the WT control. Therefore, these mutants were unlike the S1368A alteration, which was not drug specific (Mehla, et al., 2014).

**Table 5: Chemical properties of Pdr5 transport substrates used in this study a**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Aromatic rings</th>
<th>Mol volume (cm^3)</th>
<th>logP</th>
<th>H-donors</th>
<th>H-acceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerulenin</td>
<td>0</td>
<td>196.7 ± 3.0</td>
<td>0.9</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Climbazole</td>
<td>2</td>
<td>248.4 ± 7.0</td>
<td>3.3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>4</td>
<td>302.8 ± 7.0</td>
<td>5.4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0</td>
<td>247.5 ± 3.0</td>
<td>0.6</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>3</td>
<td>356.2 ± 3.0</td>
<td>7.9</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Pdr5 is known to have some substrate preferences including molecular volume (Golin, et al., 2003). The predicted values were obtained from ACD/Labs Percepta Platform via Chem Spider. ([http://www.chemspider.com/](http://www.chemspider.com/))
Figure 12: The S678Y and L1367F mutants have a drug-specific phenotype.
The S678Y and L1367F mutations were recreated in the pSS607 vector and placed in the ΔPdr5 strain, R-1. Resistance of S678Y and L1367F to clotrimazole (A), tamoxifen (B), climbazole (C), cer (D), and cyc (E) was determined in liquid YPD cultures incubated for 15 hr at 30 °C as previously described in the Experimental Procedures. In these experiments, n ≥3.
**How transport assays help identify diode mutants**

The behavior of the S1368A mutant in a series of transport assays illustrates the unique phenotype of a mutant directly involved in substrate gating (Figure 13). The S1368A mutant had a distinctive phenotype. It was severely transport deficient when R6G efflux was carried out against a concentration gradient (Figure 13A). When transport was performed in the direction of the gradient, the high concentration of R6G in the cells was diluted into external buffer without any substrate (Figure 13B), thus making reflux more difficult. Under these conditions, the S1368A mutant had a near WT phenotype in contrast to mutants that are deficient in other biochemical processes such as ATP hydrolysis or signal transmission.

Direct demonstration of reflux was observed by preloading cells with non-radioactive R6G in the absence of glucose then allowing reflux in the presence of [3H]-R6G (Figure 13C). Under these conditions, significantly more isotopes accumulated in the S1368A mutant during a short period of time (15 min).
Figure 13: Diode-deficient mutants have a distinct transport phenotype.
The behavior of WT (green arrow) and a diode-deficient mutant (violet arrow) are compared in three assays. A black arrow indicates that the mutant and WT strains behave similarly. A. When R6G transport is measured against an R6G concentration gradient, the efflux capability of the WT and diode-deficient mutant strains will be similar. However, the WT will exhibit only a small amount of reflux back into the cell in contrast to the diode mutant, which looks transport deficient because of the influx during transport. The net result is that the diode mutant phenotype will resemble that of any Pdr5 transport-deficient mutant. B. Unlike other loss-of-function mutants, however, diode mutants exhibit a near-WT phenotype when transport is carried out in the direction of an R6G concentration gradient. This is because the high concentration of R6G present inside the cell is diluted greatly upon efflux into a large volume of buffer. C. When cells are preloaded with non-radioactive R6G under de-energized conditions but allowed to transport in the presence of $[^3]$H-R6G in glucose buffer, isotope accumulation (yellow filled-in circles) due to reflux was significantly higher in S1368A mutant cells.
The S678Y mutant had impaired transport against a concentration gradient of R6G, but exhibited efflux in the direction of a gradient that was phenotypically WT. When we tested the R6G transport capability of the L1367F mutant, it was indistinguishable from the WT (Figure 14A). For that reason, this mutant was not analyzed further in this study. The S678Y mutant, however, exhibited reduced transport against a concentration gradient of 10 µM R6G in three independently grown cultures. In this series of experiments, the WT strain accumulated about 10x less fluorescence than the mutant. As expected, the S1368A mutant also showed a significant deficiency in transport. We compared the capability of the S1368A, S678Y strain to transport R6G relative to the two single mutants. Importantly, although transport by the S1368A mutant was similarly impaired, the double S678Y, S1368A double mutant had a WT transport phenotype. This finding strongly suggested an interaction between these residues as the R6G transport behavior of each single mutant shows a loss-of-function phenotype. In a second experiment (Figure 14B), the difference between the WT and S678Y strains was less pronounced, but highly significant when a two-tailed t-test was performed (p = 0.0004).

When transport along a concentration gradient was evaluated, the result was quite different (Figure 14C). The kinetics of transport exhibited by the WT and S678Y strains were nearly identical. The half-life of the retained fluorescence was about five minutes with both strains.

The S678Y mutant exhibits increased [³H]-R6G reflux during the transport cycle

We preloaded WT and S678Y cells with 20 µM R6G under de-energizing conditions. Following this, we initiated transport in the presence of 20 µM [³H]-R6G with the
addition of glucose and determined the amount of radioactivity retained by the cells after 15 minutes (Figure 14D). The mutant retained significantly more of the isotope than the WT (p= 0.0042). Taken together, the R6G transport phenotype of the S678Y mutant resembled that of the original diode mutant, S1368A and this observation suggested that both halves of Pdr5 are essential for preventing drug reflux.
Figure 14. The S678Y mutant R6G transport behavior is consistent with a gating defect. The S678Y mutant’s transport behavior was evaluated using the transport assays described in Figure 3. A. Transport against a concentration gradient of 10 μM R6G was assayed at 30 °C for 90 min in six strains as described in the Experimental Procedures (n=3). B. The assay was repeated with the S678Y and WT strains (n=3). C. Transport in the direction of a 10 μM R6G concentration gradient was evaluated with two independent cultures of the S678Y mutant and WT strains 30 °C as described in the Experimental Procedures. D. Reflux of [3H]-R6G (20 μM) during glucose initiated R6G transport was measured for 15 min at 30 °C in 0.02M Hepes, 1 mM glucose buffer (pH 7.0) as described in the Experimental Procedures (n =7).
The S678Y mutant exhibits reduced amounts of Pdr5 in purified PM vesicles

The S678Y mutant exhibits a drug-specific phenotype and was even hyperresistant to cerulenin. It also exhibits WT R6G transport properties when efflux is performed in the direction of the concentration gradient. We therefore expected that this mutant would have wild-type levels of Pdr5 in purified PM vesicles. The Western blot (Figure 15A) performed with two different preparations of PM vesicles demonstrated that the mutant exhibits a significant reduction in PM vesicle levels of Pdr5. The WT vesicles had about 3x more than the mutant. When we measured the ATPase activity of the S678Y preparations with 3 mM ATP (Figure 15B), the activity was about 25% of the WT (600.0 nmole / min / mg and 2186 nmole / min / mg respectively). We also measured ATPase activity as a function of ATP concentration. The $V_{\text{max}}$ values of the mutant and WT preparations were 0.5 and 2.9 µmoles / min / mg respectively. Thus, the reduced enzyme activity of the mutant was proportional to the reduction in Pdr5 PM vesicle levels.

Pdr5 ATPase activity is non-competitively inhibited by some of its transport substrates including clotrimazole and R6G (Golin, et al., 2007, Ernst, et al., 2008). Mutants in residues implicated in the signal interface often exhibit altered ATPase sensitivity to these compounds (Ernst, et al., 2008, Sauna, et al., 2008, Downes, et al., 2013) in contrast to the S1368A diode-deficient mutant, which did not. Similar phenotypes are observed in Cdr1 mutants that alter the signal interface (Banerjee et al. 2019). We observed that the S678Y ATPase exhibited a mild, increased sensitivity to clotrimazole inhibition relative to the WT control (Figure 15C). When a 2-way ANOVA test was performed, the curves were significantly different.
Figure 15. The S678Y mutant exhibits reduced levels of Pdr5 and its ATPase activity in PM vesicles. A. Western blotting was performed as described by Arya et al. (2019) using 5 μg of solubilized PM vesicle protein following gel electrophoresis that was performed as described in the Experimental Procedures. Two preparations each of WT and S678Y PM vesicles were used in these experiments. B. ATPase activity was measured in a reaction containing 2 μg PM vesicle protein and 3 mM ATP in Tris glycine (pH 7.5) buffer for 8 min at 35 °C in a final volume of 100 μl. C. Inhibition of ATPase activity by clotrimazole was evaluated using the same conditions described in panel B, except that varying concentrations of clotrimazole were added to different reaction tubes. The plots are the average values obtained from two PM vesicle preparations of each strain.
**The molecular diode employs the same signal transmission interface used by the**
**drug transport cycle**

Several of the suppressor mutations were recovered in residues that are in the Pdr5 signal interface (P596L) or affect the efficiency with which energy is used for translocation (A666V; see Arya, et al., 2019). Furthermore, biochemical analysis of the Tap antigen transporter clearly established that it contains a molecular diode requiring a functional D-loop (Grossmann, et al., 2014), which is critical to proper signal transmission between the sites for ATP transport and substrate translocation. Therefore, at least in the case of Tap, the molecular diode is dependent on the same signaling interface that is used for substrate transport. Based on these observations, it seemed highly likely that the same would be true for Pdr5. Nevertheless, it was important to establish whether that was the case. Furman *et al.* (2013) demonstrated that the D1042N mutation of Pdr5 in the deviant ATP-binding site D-loop uncoupled ATP hydrolysis from transport. Thus, although the ATPase activity approached a WT level, there is little R6G transport and the mutant was profoundly drug hypersensitive.

We tested the ability of the D1042N mutant to exclude $[^3]$H- R6G during transport using the reflux assay that we originally developed to characterize the S1368A mutant (Figure 16). Cells were loaded with 10 μM R6G in the absence of glucose. Transport was initiated in the presence of $[^3]$H- R6G and allowed to continue for 15 minutes. As was true in that study (Furman, et al., 2013), the WT strain accumulated very little $[^3]$H- R6G; the S1368A mutant about 3x higher (median values were 0.66 pmol / $10^7$ cells and 1.92 pmol / $10^7$ cells respectively). The reflux demonstrated by the D1042N
mutant (median value: 4.63 pmol / 10^7 cells) was comparable to that of the ΔPdr5 strain (5.03 pmol / 10^7 cells).

Figure 16. The signal interface used for drug transport is also used to prevent drug reflux. A reflux assay was performed as described in the Experimental Procedures. WT cells and cells containing a D1042N mutation were preloaded with 10 μM of R6G at 30 °C for 90 min in 0.02M Hepes buffer (pH 7.0) minus glucose before resuspension in 0.02 Hepes, 1 mM glucose buffer (pH 7.0) containing 10 μM [³H]-R6G. Transport proceeded at 30 °C for 15 min. In these experiments, n = 3.

Western blot analysis of alanine substitution mutants in the molecular diode regions

We also constructed a series of alanine substitution mutations in conserved residues that were in the gating region of the amino and carboxyl halves of Pdr5. The, G682A, I678A, F1369A, G1371A, V1372A, and M1373A mutations were made in the pSS607 plasmid and placed in the ΔPdr5 strain (R-1). We also constructed an alanine substitution in the non-conserved Cys-1370 residue. We made a single I685A as well as the I685A, V1372A double mutant.
PM vesicle preparations were made from all these strains except for the C1370A mutant, which was found to be phenotypically WT. A Western blot of the solubilized PM vesicle proteins was prepared (Figure 17). Except for the M1373A and G682A mutants, the levels of Pdr5 in the vesicles were similar to the WT. No Pdr5 protein was observed in the former and a considerably reduced amount was detected in the latter.

<table>
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<tr>
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<th>WT</th>
<th>ΔPdr5</th>
<th>F1369A</th>
<th>G1371A</th>
<th>V1372A</th>
<th>M1373A</th>
<th>I685A</th>
<th>G685A</th>
<th>I685A+V1372A</th>
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Figure 17. The M1373A mutant protein was not found in PM vesicles. A. Plasma membrane vesicles were prepared as described by Kolaczkowski et al. (1996) and modified by Ernst et al. (2008). PM proteins were solubilized in SDS PAGE and subjected to electrophoresis in 7% tris-acetate gels for 80 min at 150 V prior to staining with SimplyBlue. B. Western blotting was performed as described by Arya et al. (2019) using 5 μg of solubilized PM vesicle protein following gel electrophoresis that was performed as described in the Experimental. The Pma1 serves as loading control. Lane1: WT, Lane 2: ΔPdr5, Lane3: F1369A, Lane 4: G1371A, Lane5: V1372A, Lane6: M1373A, Lane 7: I685A, Lane8: G682A, Lane 9: I685A+V1372A.

The G1371A PM vesicle preparations have altered ATPase activities

The ATPase activities of the mutants were also evaluated. The ATPase activity of Pdr5 follows standard Michaelis Menten kinetics and representative plots are illustrated in Figure 18A. The $V_{\text{max}}$ of the F1369A mutant although on the lower end (2.4 μmole / min / mg) was nevertheless within the range of activities seen for WT activities (3.3 μmole / min / mg in the plot). The G1371A activity, however, was about half the WT
value in the two independently made vesicle preparations were tested (1.6 µmole / min / mg; Figure 18A).

When the ATPase activities of the I685A, V1372A, and double mutant PM vesicle preparations were assayed, the activities of the single mutants were all similar to the WT values (the activity of the WT preparation illustrated in panel 18B was lower than that of the preparation found in panel 18A). The double mutant appeared to have a slightly higher activity (3.8 µmol / min / mg).

We also evaluated the sensitivity of mutant ATPases to clotrimazole (Figure 18C) and R6G. The sensitivity of all the mutant ATPases resembled the WT except for the G1371A enzyme. Unlike some of the signal transmission mutants that exhibit increased resistance to inhibition by transport substrates, the G1371A mutant ATPases is remarkably hypersensitive to clotrimazole. In this regard, it was reminiscent of the Q1005H mutant described by Banerjee et al. (2020). The inhibition kinetics of the F1369A mutant generated a curve that was not significantly different from the WT when a 2-way ANOVA test was performed. This result was the same as the one obtained with the S1368A mutant (Mehla, et al., 2014).

Much to our surprise, the inhibition curves of the single I685A and V1372A mutants and the I685A, V1372A double mutant were significantly different than the WT and indicated a greater resistance of the mutant ATPase activity to clotrimazole inhibition. For instance, the double mutant enzyme had an IC$_{50}$ value of about 3.7 µM which was 2.6x the WT value of about 1.4 µM.
Figure 18. The G1371A mutant has reduced ATPase activity.  

A. The ATPase activity was measured at multiple concentrations of ATP. Assays were performed in Tris-glycine buffer (pH 9.5) with 2.5 µg of purified PM vesicle protein at 35 °C for 8 min in a circulating water bath as described in the Experimental Procedures. Representative plots are shown, and similar results were obtained with an additional preparation of WT, F1369A, and G1371A PM vesicles. B. Assays analogous to those in Panel A were carried out with PM vesicle preparations from the I685A, V1372A, and I685A, V1372A double mutant strains. C. Inhibition of ATPase activity by clotrimazole was evaluated using the same conditions described in panel A, except that varying concentrations of clotrimazole were added to different reaction tubes containing 3 mM ATP. For the F1369A, G1371A,
and the I685A, V1372A double mutant, each plot represents the average values from two PM vesicle preparations.

The F1369A and G1371A mutants were hypersensitive to all five tested Pdr5 substrates.

Using both WT and ΔPdr5 strains as controls, we evaluated the relative resistance of the mutants to the five Pdr5 substrates described in Table 2. The results for the F1369A, C1370A, G1371A, and the original S1368A diode mutant strains are found in Figure 19. Results for I685A, V1372A and the double I685A, V1372A mutant are found in Figure 20. The C1370A substitution had a WT-level of resistance to all three compounds. As expected, the M1373A mutant was phenotypically indistinguishable from the ΔPdr5 control strain when the resistance of both strains to cycloheximide and climbazole was evaluated. The F1369A and G1371A mutants exhibited resistance levels reminiscent of the original diode mutant S1368A. When 2-way ANOVA analyses were performed, the mutant plots of all five substrates were significantly different from the WT. The F1369A and G1371A mutants were more sensitive to all the drugs than the WT control, but more resistant than the isogenic ΔPdr5 control or where tested, the M1373A strain.
Figure 19. The F1369A and G1371A mutants are hypersensitive to the five tested Pdr5 transport substrates. The alanine substitution mutations were made in the pSS607 vector and placed in the ΔPdr5 strain, R-1. Resistance to five Pdr5 transport substrates was determined in liquid YPD cultures incubated for 15 hr at 30 °C as previously described in the Experimental Procedures. In these experiments, n = 3.

The I685A and V1372A phenotypes were drug specific

Bioinformatics’ analyses indicated that Val-1372 corresponds to Leu-554, which is part of the molecular plug or gate of ABCG2. Ile-685 is in a location that is analogous to Leu-555. We constructed single I685A and V1372A single mutants as well as the I685A, V1372A double mutant. We compared the relative resistance of these mutants to
the same set of five compounds used to evaluate the other mutants in this study (Figure 19). Both single mutants had striking drug-specific phenotypes. The resistance of the mutants to clotrimazole was indistinguishable from the WT (Figure 20A). Relative to the WT control, both single mutants were hypersensitive to climbazole (Figure 20B). The I685A mutant was also hypersensitive to tamoxifen (Figure 20C) and cerulenin (Figure 20D), but hyperresistant to cycloheximide (Figure 20E). In contrast, the V1372A mutant was hyperresistant to tamoxifen and cerulenin, but hypersensitive to cycloheximide. The phenotype of the I685A, V1372A double mutant, however, was broader than the single mutants. The double mutant was hypersensitive to four of the five Pdr5 substrates and for each drug had a plot that followed the trajectory of the more sensitive single mutant.
Figure 20. The I685A and V1372A mutants have drug-specific phenotypes. The single and double I685A and V1372A mutations were made in the pSS607 vector and placed in the ΔPdr5 strain, R-1. Resistance to clo (A), climbazole (B), tamoxifine (C), cer (D), and cyh (E) was determined in liquid YPD cultures incubated for 15 hr at 30 °C as previously described in the Experimental Procedures. In these experiments, n = 3.
The F1369A, G1371A and I685A mutants exhibited reduced R6G transport capability

We evaluated the ability of the alanine substitution mutants to transport against a concentration gradient of 10 µM R6G (Figure 21A). The C1370A, V1372A, and the I685A, V1372A mutants retained levels of fluorescence that were not significantly different from the WT when a two-tailed t-test was performed. However, the median retained fluorescence in the F1369A (862.0 a.u., p = 0.0012), and G1371A (3002 a.u., p = 0.0012) mutants was significantly different than the WT (89.00 a.u.) as was the retained fluorescence in the S1368A (491.4 a.u., p = 0.0011), ΔPdr5 (3865 a.u., p = 0.0001) and M1373A (4178 a.u., p = 0.0001) control strains. The level of retained R6G fluorescence observed in the I685A mutant (mean =126.5 a.u., SD = 15.09) was modestly higher than the level observed with the WT strain (mean = 96.25 a.u., SD = 10.21). Nevertheless, the variation in the independent cultures was low and a two-tailed t-test indicated the difference was significant (p = 0.019). The remaining mutants had fluorescence levels that were not significantly different from the WT.

In a second series of experiments, we compared the ability of the F1369A and G1371A mutants to transport R6G in the direction of the concentration gradient. When transport capability against an R6G gradient in the G1371A and F1369A mutants was compared to the WT (Figure 21A), the former accumulated ~30x more fluorescence; the latter 9.7x. When transport in the direction of the gradient was monitored (Figure 21C) The G1371A mutant exhibited little or no R6G transport. The F1369A mutant however exhibited transport that was statistically indistinguishable from the WT when a 2-way ANOVA test was performed.
Figure 21. The I685A, F1369A, and G1371A mutants exhibit reduced R6G transport. A. R6G transport against a concentration gradient of 10 µM R6G was assayed as described in the Experimental Procedures in experiments analogous to those in panel 4A. In these experiments, n > 4. B. The transport capability of the F1369A and G1371A mutants in the direction of 10 µM R6G was measured as described in the Experimental Procedures and is analogous to the experiment in panel 4C. The G1371A mutant exhibited significant drug reflux during transport; the F1369A and I685A mutants had the same level as the WT strain.

The G1371A mutant exhibited significant drug reflux during transport; the F1369A and I685A mutants had the same level as the WT strain.

The F1369A, G1371A, and M1373A mutants exhibited defective R6G transport when efflux was tested against and with a concentration gradient. We therefore evaluated their ability to exclude [³H]-R6G during drug transport and fully expected both mutants to have elevated levels of reflux. When glucose was added to the buffer and transport was initiated, the M1373A and G1371A mutants accumulated significantly more [³H]-R6G as did the S1368A and ΔPdr5 controls (Figure 22A). We were surprised, however, to
observe that the levels of $[^3\text{H}]-\text{R6G}$ in the F1369A and I685A mutants were statistically indistinguishable from the WT strain.

At first, this result was puzzling. However, when the relative amount of R6G accumulated against a concentration gradient (the ratio of fluorescence accumulated in the mutant versus the WT) was plotted versus the $[^3\text{H}]-\text{R6G}$ accumulated during reflux (Figure 22B), the failure to see a difference between the I685A and F1369A mutants and the WT appeared to be an issue of assay sensitivity rather than the discovery of a novel phenotype. Relative to the S1368A (which accumulates only three times more $[^3\text{H}]-\text{R6G}$ than the WT) or G1371A strains, the mutants in question were phenotypically milder with respect to the R6G transport deficiency.
Discussion

ABC transporters export substrates through a series of conformational changes. In the inward facing conformation, which is observed in multiple x-ray and cryo-electron microscopic structures, substrates bind to residues in a large pocket with relatively high affinity (Dawson and Locher, 2006). ATP binding and/or hydrolysis results in a shift to an outward facing, lower affinity conformation resulting in substrate release. It has become clear that the reduction in binding affinity is not sufficient to prevent re-entry or reflux of substrates after they have been transported out of a cell or organelle especially if the extracellular concentration is high. For this reason, it was proposed that ABC transporter has molecular diode or gate that prevent reentry of substrates (Gupta, et al., 2011). The first functional evidence for a molecular diode was described in the yeast multidrug transporter Pdr5 and the mammalian Tap antigen transporter. Mehla et al. (2014) demonstrated that a serine-1368-alanine (S1368A) mutation in Pdr5 was hypersensitive to all tested xenobiotic compounds relative to an isogenic wild-type (WT) strain even though the mutant had no defect in plasma membrane (PM) localization, drug binding or ATPase activity. However, there was considerable reflux of R6G during transport. Elegant work with the Tap transporter demonstrated a gating function that required the D-loop and presumably the same signal transmission pathway used to ferry processed antigen (Grossmann, et al., 2014).

Recently, cryo-EM structures of the mammalian transporter ABCG2 strongly implicated Leu-554 and Leu-555 as part of a molecular plug or gate (Manolaridis, et al., 2018) that separates the inner and outer binding pockets during a two-step export process. When the sequences of Pdr5 and ABCG2 are aligned, Ser-1368 is quite close to Leu-554
and Leu-555. The corresponding residues in Pdr5 are Val-1372 and Met-1373 (Figure 9A). Bioinformatics analysis of the Pdr subfamily (Lamping, et al., 2010) demonstrates that amino acids in this vicinity include Phe-1369, Gly-1371, Val-1372, and Met-1373 are highly conserved (Figure 9B). A similar set of residues is found in the amino terminus of Pdr5 and extends from Tyr-680 to Ile-685 (Figure 9C).

In the current study, the conserved set of amino acids (1369-1373) in the carboxyl-terminus motif of Pdr5 as well as the corresponding residues (680-685) in the amino terminus were tested to determine the role of these residues in diode function.

Results from the transport experiments and biochemical assays plus recent structural data suggest that a set of contiguous, conserved amino acids present in the fungal, Pdr subfamily have different roles in the gating process. These residues are also maintained in the mammalian ABCG subfamily and likely play similar roles. Assuming the actual gate is composed of Ile-685, Val-1372, Met-1373, it is plausible that Ser-687, Ser-1368, and Phe-1369 provide structural support. Alanine substitutions in these residues exhibit the unique gating-defect phenotype. Their transport is similar to the WT when R6G transport is monitored in the direction of a concentration gradient. When these mutants are forced to efflux R6G against a concentration gradient, however, they show a significant transport deficiency. Gly-1371, however, appears to play a distinctly separate role. The G1371A mutant is deficient in R6G transport both with and against a concentration gradient of R6G. Furthermore, its reduced ATPase activity in two preparations of PM vesicles and the ATPase activity's extreme hypersensitivity to inhibition by clo suggests a role in communicating signal to the gate region.
The croEM image of Pdr5 suggests that the actual gate is composed at least in part by Ile-685, Val-1372, and Met-1373. Our characterization of these residues yielded several observations. As was the case with the L555A mutant of ABCG2, the equivalent Pdr5 mutant (M1373A) was not found in purified membrane vesicles. As expected, its phenotype was indistinguishable from the isogenic ΔPdr5 control. This suggests that in addition to its gating function, this residue plays a critical role in protein folding that is evolutionarily conserved at least in the ABCG / Pdr subfamilies of ABC transporters. It is therefore reminiscent of the conserved Phe-508 residue in the CFTR chloride channel. Roughly 70% of all cystic fibrosis patients lack this residue, which results in a protein that misfolds and never reaches the membrane (Lukacs, et al., 1993). Mutations in the corresponding position of both the yeast Yor1 multidrug transporter and P-glycoprotein have similar phenotypes (Katzmann, Epping, and Moye-Rowley, 1999, Loo, Bartlett, and Clarke, 2002).

The analysis of the I685A and V1372A mutants is also instructive. The single mutant phenotypes are relatively mild and drug specific. For instance, the V1372A mutant exhibits significant, but mild cycloheximide hypersensitivity, but R6G transport is indistinguishable from the WT. The I685A mutant has a phenotype that is almost exactly the opposite. It would therefore appear that the chemistry of the compound is important in the interaction of the transport substrate and the molecular gate. It is; however, not clear which chemical parameters are important. There is no obvious correlation between phenotypes and the chemical features found in Table 1. One possibility, however, is that the molecular geometry of the transport substrates plays a role. We have not modeled these compounds in that respect. Furthermore (excluding the misfolded M1373A
protein), both gate residues had phenotypes that were less severe (and less broad) than either the S1368A or F1369A mutants. This suggests that some of the residues making up the gate have functional overlap.

Perhaps the most puzzling feature of this study was the observation that the ATPase activity of the gate residues mutants (I685A, V1372A and the double I685A, V1372A mutant) was more resistant to clotrimazole inhibition than the WT. One plausible explanation is that these residues are also part of a non-competitive inhibition site. This is consistent with prior observations (Golin, et al., 2007). When non-competitive inhibition of the Pdr5 ATPase by clotrimazole was first investigated, we entertained the possibility that the inhibition site and the transport site were equivalent. An experiment designed to test the hypothesis strongly indicated that this was not the case. We also showed that clo was not acting directly at the ATP-binding sites. These studies indicated that the inhibition site was elsewhere. Gupta et al. (2011) proposed that this inhibition was a way to freeze the transporter in the outward facing conformation and prevent reflux when the extracellular level of inhibiting drug was high (in part due to successful efflux). If the inhibitory site includes the gate, the two drug exclusion mechanisms would be coupled.

Studies with both P-gp and Sav1866 suggest that the switch from the inward-facing, drug-binding conformation to the outward-facing, drug-releasing conformation result in roughly an order of magnitude decrease in affinity (Sauna and Ambudkar, 2000). The reflux studies described here and in our previous report (Mehla, et al., 2014) suggest that the presence of a WT gate adds almost another order of magnitude to drug exclusion.
An alternative explanation for these results is that these mutants identify residues that are also involved in the signal interface. This seems less likely. Although the ATPase activity of these mutants is more resistant to clotrimazole, these strains don’t show altered resistance to this substrate. In contrast, signal-defective mutants such as S558Y and V656A, which have clotrimazole-resistant ATPase activity, are hypersensitive to this substrate.

The suppressor screen was valuable in identifying Ser-678 as important for gating function. It is also interesting that in addition to S678Y, the L1367F mutant appeared in two independent cultures. In all three mutants, the substitution resulted in a side chain that was significantly larger. This suggests that residue size at position 1368 may be critical. The original S1368A mutant resulted in a smaller residue and perhaps this was compensated for by the tyrosine and phenylalanine substitutions. The A1368V revertant is also consistent with this idea.

It is worth comparing the suppressor screen used in this study to the two others conducted previously with Pdr5 (Sauna, et al., 2008, Ananthaswamy, et al., 2010, Downes, et al., 2013). In both earlier studies, the suppressed mutations were in the signal interface regulating both the drug transport and gating steps. Virtually all the second-site mutations were also in interface. In the present study, the suppressors of S1368A mutant hypersensitivity fell into multiple biochemical processes. The S678Y, L1368F, and S1368V mutations were in the diode region, but the P596L mutant was in the signal interface. The A666V mutant was in a residue known to regulate transport binding site cooperativity. An A666G mutant increased drug resistance by increasing the transport
site cooperativity. An A666V mutant made in that study had the same phenotype (Arya, et al., 2019).

Intragenic suppressors often identify residues that interact with each other in the same biochemical step. ABC transporters, however, are polytopic proteins with multiple biochemical steps. For instance, Pdr5 has ATPase activity, intradomain signaling, drug binding/transport, and molecular gating. It is useful to view these as a series of dependent or epistatic interactions between steps much as classical geneticists and biochemists used to view interactions between gene products. Thus, for example, the relatively early steps of ATP hydrolysis and intradomain signaling are essential for proper gating. A defect in signaling would not be remedied by a mutation in a gating residue, which involves a relatively later biochemical operation. Therefore, idea of biochemical epistasis explains why virtually all the suppressors of the signal defective S558Y and N242K mutants lie in that early biochemical step, but suppressors of gating defects appear in multiple biochemical steps. For instance, consider A666V suppressor of S1368A. Even though the original leak remains, the presence of the A666V mutation affects cooperativity between transport sites and ensures that more substrate is pumped per unit time thus mitigating the gating defect (Arya, et al., 2019).

**Future directions**

The research described in this dissertation that a group of highly conserved residues in both halves of Pdr5 are required to prevent reflux of drugs following transport out of the cell. CryoEM studies by our collaborators clearly show that Ile-685, Val-1372, and Met-1373 make up the gate proper. Our experiments indicated that Ser-678, Ser-1368, and Phe-1369 are also required for gating and probably lend additional
structural support. A similar set of residues with analogous function were observed in the structurally related, mammalian transporter, ABCG2.

A major question that remains is whether this set of residues is present in other ABC transporter subfamilies and to what extent these residues functionally analogously. For example, the mammalian ABCC subfamily contains several multidrug transporters such as Mrp1 and Mrp3 (Cole, et al., 1992). In yeast, the Yor1 multidrug transporter is a member of this subfamily. It was initially identified as an oligomycin efflux pump (Katzmann, et al., 1995). An alignment with Pdr5 indicates that while the diode residues in the carboxyl terminus are conserved in the ABCC subfamily, those in the amino terminus portion are not.

It would be important to do mutational analysis of Yor1 to determine whether the analogous residues define a molecular gate. An approach similar to the one used to characterize Pdr5 mutants seems reasonable. However, Yor1 has not been extensively studied biochemically. For instance, R6G is not a Yor1 substrate, so other fluorescent compounds that are transported by Yor1 would need to be identified. The ATPase activity of this transporter has not been extensively characterized and development of a good assay would be essential for mutant analysis. Unlike Pdr5, Yor1 basal ATPase activity appears low so drugs that stimulate activity would probably need to be identified. Many Yor1 substrates are known so this should be straightforward. Once these experimental prerequisites are met, alanine-scan mutagenesis would be performed with the carboxy-terminus residues. If, when characterized, these alanine substitutions behave as gating-deficient mutants, suppressor screens that restore drug resistance might identify amino-terminus residues that are also required for proper diode function.
References


